# STUDIES OF GIARDIA LAMBLIA

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ABSTRACT

Title of Dissertation: Studies of *Giardia lamblia*

H.D. Alan Lindquist, Doctor of Philosophy, 1994

Thesis directed by: John H. Cross, Ph.D., Professor, Department of Preventive Medicine and Biometrics

Three different aspects of the biology of the parasite *Giardia lamblia* were addressed. They were: the ability of different strains of *G. lamblia* to elicit differential delayed type hypersensitivity responses in mice, the development of a hybridoma cell line which produced antibodies to a 47.7 kd antigen of whole trophozoites, and the development and characterization of *G. lamblia* resistant to a 9 micromolar concentration of albendazole for 24 hours. Albendazole has been suggested as an alternative therapy to the currently used chemotherapeutic agent, metronidazole, since *G. lamblia* is increasingly resistant to metronidazole. It was found that resistance was reversible over time, such that a strain resistant to an exposure to 9 micromolar albendazole for 24 hours could show a resistance reduced to a maximum of 7 micromolar albendazole for 24 hours after as little as two weeks without re-exposure to the 9 micromolar dose of albendazole. When culture supernate containing drug was incubated with resistant trophozoites, it retained its ability to kill susceptible trophozoites. The resistant

trophozoites retained their ability to encyst. There was no resistance to metronidazole conferred by albendazole resistance. Ultrastructural examination of resistant trophozoites after a 24 hour exposure to 9 micromolar albendazole showed that resistant trophozoites were able to maintain a structurally sound striated disk, while susceptible trophozoites showed degradation of the striated disk. Uptake of $^{14}C$-labeled albendazole showed that the resistant trophozoites took up more of the radiolabeled drug within the first 38 hours of exposure. Albendazole resistance in the laboratory model is probably due to a change in the target of action of the drug, requiring a greater amount of drug to act against this target to effect killing of the trophozoite.

Studies of *Giardia lamblia*

by

H.D.Alan Lindquist

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**Introduction:**

Although much is known about *Giardia lamblia*, its epidemiology, biology, molecular biology and immunology, the mechanism of pathogenesis is largely unknown. For example, the contribution of the host immune system to pathogenesis is as yet undefined. Nash *et al.* (1987) showed that a pathogenic isolate of *G. lamblia* was not pathogenic to all of the individuals experimentally tested for infection: therefore it can be surmised that some host factors interact with pathogenicity. Current methods of diagnosis are frequently ineffectual. Typically a stool exam should be repeated three times on alternating days in order to give a high probability of detecting light infections. Other methods of detection of *G. lamblia* are either unreliable or are not field transportable. Finally, *G. lamblia* has demonstrated reduced sensitivity to metronidazole, the drug of choice in treatment, necessitating a search for new drugs, and an evaluation of the potential for *G. lamblia* to develop resistance to these new drugs.

*Giardia lamblia* is a protozoan parasite of humans and other animals and is distributed throughout the world. *Giardia lamblia* was first described from a diarrheic human stool (Meyer, 1994). The disease associated with infection by *G. lamblia* is known as giardiosis and is characterized by a variety of symptoms, including: diarrhoea, weakness, weight loss, abdominal pain, nausea, steatorrhoea, flatulence, vomiting, and fever. Any one individual may

exhibit a combination of some or all of these symptoms, and there are many cases of asymptomatic infection (Farthing, 1994). Giardiosis is usually non-fatal. *Giardia lamblia* is transmitted by water, food, hand to mouth, and sexual routes.

History:

Levine (1979) reported that trophozoites may have first been seen by Antony van Leewenhook in 1681 in his own diarrhoeic stool using his primitive microscope. Members of this genus were assigned to the genus *Cercomonas* with the trivial name *intestinalis* by Lambl in 1859. This name was a homonym of another organism which had been referred to the genus *Cercomonas* by Deising in 1850 previously known as *Bodo intestinalis*.

In the biographical abstract appended to an article by Lesh (1975), Losch (Lesh) is said to have described the parasite from a patient's stool in 1870. In 1882, Kunstler contributed the generic name *Giardia* to the species isolated from tadpoles as a tribute to the French microscopist Giard. In 1888, Blanchard named the genus *Lamblia* in honor of Lambl using a species isolated from mammals. Although Blanchard had been preceded by Kunstler, the genus *Lamblia* is still used in some parts of the world, most notably eastern Europe.

In 1881, Grassi named the species *Megastoma entericum* applying the name to parasites possibly isolated from several hosts. Stiles, in a 1915 letter to Kofoid, proposed that the specific name *lamblia* be used to avoid the confusion between the specific names *intestinalis* and *enterica*. The species name *lamblia* was formally proposed by Kofoid and Christiansen in 1915. The rationale was that Grassi had changed the trivial name *muris* to *entericum* in 1879 to denote *Giardia* isolated from a mouse. Corradetti (1935) pointed out that the title of Grassi's 1881 paper specifically denoted *Megastoma entericum* as being a new parasite of man and therefore proposed *entericum* as the appropriate trivial name for *Giardia* of man.

The concept of host specificity as a criterion of speciation was noted by Filice (1952) as having been used by Hegner in a 1926 publication and this concept was adopted by others. He also noted that Nieschultz in 1924 had refuted this concept and divided the many species into sub-genera on the basis of major morphological features, especially the median bodies. Filice (1952) defined *Giardia* of man and other indistinguishable parasites from animals as *Giardia duodenalis* (Davaine, 1875) which Davaine had originally described as *Hexamita duodenalis* of rabbits.

Currently, the most common designations for *Giardia* of man are: *Giardia duodenalis*, *G. enterica*, *G. intestinalis*, *G. lamblia*, and *Lamblia duodenalis*.

Perhaps the simplest and most biologically relevant method of determining species is to accept species separation on the basis of major morphological variation, in conjunction with a limited degree of host species specificity. With this paradigm, there would be at least four major species of *Giardia*: *Giardia muris* (Grassi, 1879) of mice and rodents, *Giardia agilis* (Künstler, 1882) of frogs, *Giardia ardea* (Nöller, 1920) of great blue herons, *Giardia psittaci* (Erlandsen and Bemrick, 1987) and *Giardia lamblia* (Lambl, 1859) of humans and a variety of other animals. These species may be differentiated by microscopic morphological characteristics.

Classification:

*Giardia lamblia* (Stiles, 1915)(syn. *Giardia intestinalis*, *Giardia duodenalis*, *Giardia enterica*, *Lamblia duodenalis*) is classified in the kingdom Animalia or animals as it lacks a cellulose cell wall or the other characteristics of plants. It has been placed into the subkingdom Protozoa, the single celled animals. It is in the phylum Sarcomastigophora which includes those protozoa with flagella and pseudopodia. *Giardia lamblia* is placed in the subphylum Mastigophora, which includes only flagellates. The lack of chloroplasts allows classification in the class Zoomastigophora. Since *G. lamblia* has two karyomastigonts and flagella arising near the nucleus it is classified in the order Diplomonadida. *Giardia lamblia* has eight flagella and is subsequently placed in the family Hexamitidae which

includes organisms with six or eight flagella. This classification follows the scheme of Beaver *et al.* (1984).

Morphology:

*Giardia lamblia* has two life cycle stages, the trophozoite and the cyst. Trophozoites have a characteristic pear shape with a convex dorsal surface, and concave ventral surface. Trophozoites have a range from 10-20 µm in length, 5-15 µm in greatest width, and 2-4 µm in thickness. Morphological features of *G. lamblia* readily observable by light microscopy include four pairs of flagella, two nuclei, and two central axonemes arranged in rough bilateral symmetry. There is a ventral disk which is concave and appears to be a sucker-like, or a suction cup-like organelle. It is striated on electron micrography and hence has been called by some authors the striated disk (Jones *et al.*, 1983). The striated disk is composed of a central area enclosed by a concentric band of microtubules and associated ribbons of proteins which are joined by protein bridges, and bounded by an edge of actin and actin-like proteins called the lateral crest. The disk structure is surrounded by a flange of cytosolic material which overhangs the boundary of the disk and is termed the ventro-lateral flange.

Other internal structures of *G. lamblia* trophozoites include: karyomastigonts, a funis, and median bodies which

in *G. lamblia* appear hammer claw shaped. These structures are comprised of microtubules. The karyomastigonts are located between the nuclei and form the origin of the flagella. The funis is located dorsal to the striated disk and may be an organizing center for the microtubular structure of the parasite. The median bodies, located posterior to the nuclei and crossing the central axonemes, may also be important to the organization of the trophozoite. Membrane bounded structures include vacuoles which are probably pinocytotic in origin, and endoplasmic reticula (Feely *et al.*, 1990).

The cyst is the environmentally resistant stage of the *G. lamblia* life cycle. This enables the parasite to survive outside the host as a cyst until it is swallowed by another suitable host. In the cyst stage the parasite is relatively quiescent, however respiration still occurs at a low level (Gillin *et al.*, 1989, Kasprzak and Majewska, 1983, Sauch *et al.*, 1991). The cyst has a tough fibrous outer wall and measures from 8-19 µm in length by 7-10 µm in diameter, is ovoid in shape and contains 4 nuclei, each with an eccentric karyosome. Some remnants of other ultrastructural components of the trophozoite may be found in the cyst, often including prominent axonemes (Feely *et al.*, 1990).

In Nomarski differential interference contrast (DIC) microscopy there are two types of cyst readily observable

for *G. lamblia*[1]. Type I cysts have a refractile, smooth and oval shaped cyst wall with a visible axostyle and median body (Gillin *et al.*, 1989). Type II cysts have index of refraction similar to that of water and the internal structure is less defined. By comparison on the basis of exclusion of propidium iodide, incorporation of tritiated thymidine and by direct observation of *in vitro* excystation, type I cysts have a higher probability of excystation.

Life cycle:

The life cycle of *G. lamblia* is simple when compared to most other human parasites. The trophozoite form is located in the lumen of the upper part of the small intestine and colonizes the exterior of the microvilli and the brush border of the intestinal columnar epithelium. Trophozoites are found in the upper small intestine. Since vitamin B-12 is absorbed in the ileum, vitamin B-12 deficiency commonly seen in giardiosis is evidence that trophozoites may also be located in the ileum (Wright *et al.*, 1977). Localization may be established by the use of

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[1] Nomarski DIC is achieved by placing a polarizing filter between the light source and the object to be viewed. A second polarizing filter in introduced into the light path between the objective lens and the ocular oriented so the direction of polarization is 90 degrees opposed to the substage polarizing filter so that the light is at extinction. An analyzing filter is then interposed above the objective. A lambda filter may be imposed in the ocular tube to visualize the field in color.

lectins on the surface of the trophozoite, which may preferentially recognize some marker in these locations (Jones *et al.*, 1983). Localization is maintained by the active motility of the trophozoite, and also by the trophozoite's streamlined shape, its low profile when attached, and by its ability to attach to the brush border of the intestinal columnar epithelium. When trophozoites begin to pass down the intestinal tract, encountering an increasing concentration of bile salts, they encyst. It has been shown *in vitro* that encystation occurs in the presence of certain specific bile salts and lactic acid (Gillin *et al.*, 1989).

The striated disk on the ventral surface of the trophozoite is associated with attachment of the trophozoite to the intestinal epithelium. Scanning electron micrographs of intestinal epithelial cells show deformations of the microvilli corresponding to the outline of the ventral disk of the trophozoite (Erlandsen, 1974). The mechanism of attachment of *G. lamblia* might be through contraction of the edge of this striated disk where the actin fibrils are located. In this model, the concentric band of microtubules would provide structural support.

A second proposed mechanism of attachment relies on a pair of flagella which originate on the ventral side of the organism near the center of the striated disk. There is a groove in the ventral aspect of the trophozoite, in which

this pair of flagella lie. The action of these flagella may create low pressure under the area enclosed by the striated disk relative to the outside pressure, thereby creating a pressure differential by which the trophozoite may remain attached to the substrate, intestinal epithelium *in vivo*, or culture tube *in vitro*. This theory helps explain why trophozoites can remain attached to glass and other unyielding substrates perhaps better than the theory that through contraction the edge of the disk grasps the substrate.

In any event, the end result is that the trophozoite is attached to the substrate by close apposition of the ventral striated disk and the substrate. The striated disk is therefore considered an organelle of attachment.

*Giardia lamblia* is an interesting target for study because it represents a "missing link" in the evolution of eukaryotic organisms. The small subunit ribosomal RNA (SSUrRNA) of *G. lamblia* is more similar in size to that of the archaeobacteria, than to that of other, supposedly related, eukaryotes. Since the SSUrRNA is usually highly conserved between related species, this indicates that *G. lamblia* may represent a transitional stage between the archaeobacteria, which share a similar SSUrRNA, and the eukaryotes which share the characteristic of a nuclear membrane. As an evolutionary transition stage organism, the

biology of this species presents an interesting target for a study as the biological processes which may elucidate the differences between the two groups which *G. lamblia* bridges (Sogin *et al.*, 1989).

Immediately before encystation the trophozoites undergo nuclear replication producing a cyst with four nuclei. Although the cyst is quiescent, there is evidence that respiration continues to occur and viable cysts may be able to exclude certain dyes such as eosin (Kasprzak and Majewska, 1983) and propidium iodide (Sauch *et al.*, 1991). Cysts survive in the environment especially in cool moist environments. Knowledge of a probable series of events upon ingestion is given by *in vitro* excystation experiments (Bingham and Meyer, 1979, Coggins and Schaefer, 1984, 1986, Schaefer *et al.*, 1984, Buchel *et al.*, 1987). Most *in vitro* excystation protocols utilize a pre-excystation step in acidic medium, simulating passage through the stomach, followed by a rapid increase in pH mimicking passage into the small intestine. The rapid changes in pH may trigger excystation. In some protocols the addition of trypsin or some other enzyme may help weaken the cyst wall. The proto-trophozoite emerges from the cyst as an amorphous mass with four nuclei. This mass divides with two resultant progeny each receiving two nuclei and the cytoplasmic material being distributed between the two progeny (Schaefer, 1990).

Epidemiology:

*Giardia lamblia* is among the most frequently diagnosed protozoan parasites of humans in the United States. It is distributed globally, with some areas being endemic, while other areas are prone to epidemics. The distribution of cases in the population consequently varies between endemic and epidemic areas.

In endemic areas, children are most often infected, while in epidemics the age distribution is dependant on the source of the outbreak. In endemic areas adults usually have a lower rate of infection than children. In non-endemic areas, more adults may be infected. The low infection rate for adults in endemic areas may be due to an incomplete immunity.

Frequently, the incidence of new *G. lamblia* infections is seasonally distributed. Seasonality is likely a result of increased transmission probably due to increased human contact with sources of infection.

A New Hampshire study (Dennis *et al.*, 1993) of endemic giardiosis demonstrates this seasonal distribution. The seasonality seen in New Hampshire was attributable to an increased host/parasite contact when the onset of warm weather coincided with increased outdoor activities leading to increased exposure to possible sources of infection, such as swimming in lakes, and drinking untreated surface water. In other areas, the seasonal incidence may be due to other

exposure-related factors such as rainfall which might wash contaminants from the surface into exposed wells or cisterns.

Numerous outbreaks of giardiosis have occurred in the United States, due to contaminated water sources, sexual transmission, and to food-borne outbreaks (Owen, 1984). Large waterborne outbreaks of *G. lamblia* have forced public health departments to issue "boil water" orders. These orders are directives from the local health department warning that the local tap water is unsafe for human consumption and must be rendered safe by boiling. Purchasing water from a reliable source is also recommended.

It is difficult to prove that a water system is contaminated with *G. lamblia*. A drinking water pre-treatment system (a water treatment system meant for the purification of water before delivery to the end users as opposed to water utilities which use water from deep wells or aquifers), which allows the passage of *G. lamblia*, may still pass microbiological purity standards for fecal coliform bacteria. Specific microscopic examination for *G. lamblia* cysts may prove that a water system is contaminated, but a negative examination does not conclusively prove that the system is not contaminated. Depending on the type of pre-treatment system used, it is possible to contaminate drinking water by mismanagement of the pre-treatment system. Once found to be contaminated, it is very difficult to certify that a public water supply is *G. lamblia* free. No

test exists which is 100% effective in recovering *G. lamblia* cysts from water. Since only one cyst is theoretically necessary to start an infection, current technology can only indicate that there is a low probability of *G. lamblia* contamination, and not that a water supply is *G. lamblia* free. Contamination of a water supply may lead to expensive remediation efforts by the utility providing the water, which may increase utility cost, or taxation.

Pathology:

The variability of symptoms in giardiosis led some investigators to conclude that *G. lamblia* was non-pathogenic. An example of this occurred when Rendtorff (1954) exposed prison inmates to *G. lamblia* cysts to determine transmissibility. At the time, while *G. lamblia* was used as a suitable non-pathogenic protozoan model for amoebae, it was considered unethical to knowingly expose prisoners to *Entamoeba histolytica*. In 1979 Rendtorff wrote that in retrospect the strain of *G. lamblia* used was non-pathogenic, and that the opinion in the 1950's was that *G. lamblia* was a non-pathogenic organism. This theory flourished for many years, but in the 1980's a controversy arose as to the ability of the parasite to cause clinical symptoms.

In 1987, Nash *et al.* (1987) put this argument to rest. They inoculated human volunteers with several pathogenic strains of the parasite of human origin,

producing symptoms in two of five volunteers who had been inoculated with a pathogenic strain. Koch's postulates were therefore satisfied. It is now felt that *G. lamblia* is a cause of clinical illness in humans.

The minimum infective dose of *G. lamblia* is 1-10 cysts. The prepatent period for infection is 10 to 36 days depending on the dose, with smaller doses requiring longer times until patency (Rendtorff, 1954).

Giardiosis is characterized by a variety of symptoms. In the United States, especially in adults, it is frequently described as a diarrhoeal illness. The diarrhoeic stool may be frothy, foul smelling, and steatorrhoeic. Diarrhoea may be accompanied with cramps, headache, bloating, borborygmy, flatulence, and anorexia. However any of these signs or symptoms may be absent in a particular individual. Damage to the structure of the intestinal epithelium has also been documented (Zamcheck *et al.*, 1963) in which the villi are shortened, the villous to crypt ratio decreased, the rate of division of epithelial cells in crypts increased, and the number of goblet cells increased.

Systemic symptoms have been reported including arthritis (Woo and Panayi, 1984) urticaria (Hamrick and Moore, 1983), hypokalemic myopathy (Cervello and Alfaro, 1993) and chronic fatigue syndrome (Levine *et al.*, 1992), although less common than diarrhoea, may be indicative of

the interaction of *G. lamblia* with the immune system, or may imply an underlying condition of the host.

Acute diarrhoea and gastroenteritis due to *G. lamblia* will generally resolve without treatment in about two weeks. Without treatment, the individual: (1) may become a chronic host of *G. lamblia* with long term chronic symptoms, (2) may begin to pass cysts asymptomatically, or (3) may clear the infection.

*Giardia lamblia* has been implicated in causing "failure to thrive" syndrome (failure to grow at a normal pace) in children of the third world (Gupta and Mehta, 1973). Children with symptomatic infections may also grow in height at a slower pace than uninfected children (Farthing *et al.*, 1986). In the third world, anorexia and failure to thrive in children may be the main clinical features of giardiosis. Diarrhoea and the other symptoms described above may also be present.

Generally, children in developed countries do not suffer from failure to thrive from giardiosis. The differences in the symptoms between third world children and adults, and adults in developed countries may indicate differences between the underlying nutritional and immune status of the individuals.

Research undertaken:

Three distinct studies were undertaken within the scope of this investigation. The first study was designed

to determine if there were differences in strains of *G. lamblia* with respect to the ability to cause delayed type hypersensitivity (DTH). The exact mechanism of pathogenesis of giardiosis is unknown; however the pathology seen in some cases is consistent with a cell mediated immune (CMI) response of a host against its own intestinal epithelium. Furthermore, there is at present no sure way to determine if a strain or isolate of *G. lamblia* is pathogenic.

Many methods have been used to attempt to differentiate strains, including isoenzyme studies, restriction fragment length polymorphism, random amplified primer analysis, and others. None of these methods has allowed for reliable differentiation of pathogenic and non-pathogenic strains. Given these facts, a difference in the ability of strains to cause DTH in an animal model would lend credence the hypothesis that DTH might be involved in the pathology of giardiosis. A differential DTH response might also be a biologically relevant character of *G. lamblia* strains which would be predictive of pathogenicity.

The intent of the second study was to develop a dipstick antigen detection test which would be easily field transportable. This required the development of hybridoma antibody producing cells specific for *G. lamblia* antigens.

There are several methods for detection of *G. lamblia* infection currently in common use. First there are microscopic methods. Direct microscopic examination of concentrations from stool, examination of stool specimens

concentrated with some technique such as zinc sulfate, and the microscopic examination of intestinal contents withdrawn with a string capsule are common techniques. These may be supplemented by fluorescent conjugated antibody techniques specific for *G. lamblia*. However these techniques require access to a fluorescent microscope and trained microscopist. An enzyme linked antigen capture technique is also commercially available. Unfortunately this test is not conveniently transportable to the field, and may produce an unacceptably high false negative rate.

A dipstick test for *G. lamblia* diagnosis would save time and resources and should be accurate and easy to read. It would also be easily transportable to the field for field diagnosis. A dipstick would consist of a nitrocellulose strip affixed to a backing and prepared with a spot of antibody which would acquire *G. lamblia* specific antigens from a stool sample. A second antibody would then be used to recognize these captured antigens, and a recognition system such as an enzyme linked antibody used to detect binding of the second antibody. Dipsticks have the advantage of being able to incorporate a positive control on the same test strip as the sample, and are more readily transportable than other available diagnostic tests.

The third study was to develop a *G. lamblia* strain which was resistant to the drug albendazole. This is important as albendazole is being considered as a possible

alternative to metronidazole in the treatment of giardiosis. It is, therefore, necessary to predict whether it is likely that *G. lamblia* will develop resistance to albendazole. It is important to determine if the mechanism of resistance to albendazole is similar to that in metronidazole resistance. The ability to induce resistance to albendazole may also serve as a model for testing the prospects for long term efficacy of drugs proposed for use in the treatment of giardiosis.

**Delayed Type Hypersensitivity and Strain Differences:**

**Introduction:**

The mechanism of *G. lamblia*'s pathogenesis is incompletely understood. Light and electron photomicrographs show that the parasite causes mechanical displacement of microvilli and may on occasion penetrate the cells of the intestinal epithelium although penetration of the epithelium may be an artifact of preparation of the specimens (Ganguly *et al.*, 1985, Morecki and Parker, 1967, and Saha and Ghosh, 1977). Radulescu *et al.* (1980) showed that *G. lamblia* in culture with human fibroblasts was cytopathogenic, and this may be the means through which the parasite causes its characteristic pathology. Another study, using kidney cells to replicate the reaction of an active epithelial cell layer, showed no invasion of the cell layer and basically no cytotoxic effect by *G. lamblia* (Chavez *et al.*, 1986). Studies of the intestinal epithelium show that in some cases *G. lamblia* infection causes stunting of the villi, reduction of the villous to crypt height and an increase in cell replication at the base of the villi (Yardley *et al.*, 1964). This pathology may also be present in asymptomatic children and may not be present in some symptomatic cases, and therefore stunting of the villi may not be the sole cause of the symptoms observed.

Intestinal epithelial cells are derived from dividing cells at the base of the villi, moving toward the

tip of the villi as cells at the tip are sloughed off. This movement brings them into contact with the parasites in the lumen of the intestine. In the mouse model, division at the base of the villi is increased (Ganguly *et al.* 1985). Mechanical damage by *G. lamblia* does not appear to be responsible for the loss of cells at the tips of the villi because the *G. lamblia* cause only minimal damage to these cells in *vivo*, although as stated before *G. lamblia* in culture is cytopathogenic to human fibroblasts. The reason for the loss of these cells, and subsequent and overall stunting of the villi may be an auto-immune reaction. Sloughing of cells may not be the only, or even the primary mechanism of pathogenesis. Changes in the villous architecture are not always seen, even in symptomatic patients. However, the reasons for these changes in villous architecture represent a good target for investigation, which would lead to a better understanding of pathogenesis.

In the mouse model, the parasite shows a predilection for regions of the lumen containing Peyer's patches (Gillon *et al.*, 1982, Owen *et al.*, 1979, and Owen *et al.*, 1981). These patches are rich in immune cells which may be responsible for mounting an immune response to intestinal infections. The association of *G. lamblia* with these areas is suggestive of an adaptive event in the evolution of the host-parasite relationship. Close association of *G. lamblia* with immune cells in the Peyer's

patches would be expected to elicit an immune response from the host which is protective or advantageous to the *G. lamblia* by some unknown process. Perhaps the symptoms of gastroenteritis are adaptively selected by virtue of providing a better dispersion of *G. lamblia* in the environment, or there may be some protective benefit to *G. lamblia* in the lumen by removing potential *G. lamblia* toxins from the lumen rapidly.

Cyst excretion in albino Swiss mice was greater when those mice were inoculated with isolates from patients with severe diarrhea, and cyst excretion decreased with inocula from patients with a decreasing severity of symptoms (Aggarwal *et al.*, 1983). That the immune system response plays a role in cyst excretion in albino Swiss mice was reported by Aggarwal *et al.* (1980). They showed that mice injected with corticosteroids, or subjected to irradiation showed greater cyst excretion than controls infected with the same parasite isolate. This does not address the problem of the severity of symptoms.

*Giardia lamblia* elicits antibody production of both IgG (Smith *et al.*, 1981) and secretory IgA (Nayak *et al.*, 1987) in infected persons. The role of these antibodies is probably parasite clearance and prevention of re-infection. *Giardia lamblia* may cause prolonged infections in strains of mice which are T cell deficient (Roberts-Thomson *et al.*, 1978). The current hypothesis of humoral immunity is that it mediates parasite clearance and immunity to re-infection

with homologous strains. Humoral immunity does not appear to be related to pathogenesis. If thymectomized mice are reconstituted with T cells they show higher villous to crypt ratios (Roberts-Thomson *et al.*, 1978). Taken with the information on humoral immunity, the implication is that cell mediated immunity (CMI) may play a role in pathogenesis.

*Giardia lamblia* is capable of stimulating macrophage and mononuclear leukocytes to become non-specifically cytotoxic to both *G. lamblia* and other "innocent bystander" cells (Smith *et al.*, 1982). This phenomenon may be responsible for the sloughing of villous epithelial cells. The current model for the elicitation of CMI is that a cell must be infected with a foreign antigen such as a virus before it can present that antigen in the context of major histocompatibility class (MHC) I proteins. After presentation in the context of MHC I proteins the infected cell is then killed by the CMI response. Epithelial cells may then internalize some parasite antigen and present it in the context of MHC I proteins, thus eliciting a CMI response. This response may be responsible for killing these cells, resulting in the sloughing, and the stunting of the villi. To determine the immune system's mechanism of clearance of *G. lamblia*, it is first necessary to determine if this parasite elicits a CMI response which may be responsible for symptoms.

Close association of *G. lamblia* with intestinal epithelial cells may result in the epithelial cells presenting giardial antigens in the context of MHC proteins as though they were infected by an intracellular parasite, thus eliciting a CMI response which results in the epithelial cell being killed. This response may result in killing and sloughing of epithelial cells leading to symptoms of gastroenteritis. Parasites which elicit a lower level of CMI may have a better opportunity for survival and reproduction. Conversely, parasites eliciting a higher level of response may proliferate more rapidly, shed into the environment and have a higher probability of being transmitted. Therefore, the CMI response may be integrally related to both the severity of symptoms and the transmissibility of giardiosis.

Isolates of *G. lamblia* have been subdivided into strains on the basis of restriction endonuclease analysis (Nash *et al.*, 1985), excretory secretory products and surface antigens (Nash and Keister, 1985) drug resistance (Majewska *et al.*, 1991) and isoenzyme analysis (Abaza *et al.*, 1991). While Nash *et al.* (1985) reported that restriction endonuclease analysis gave consistent results, it was noted that the results were surprising in that a beaver from Canada, and patient isolates from Afghanistan, Ecuador, Puerto Rico, and Bethesda, Maryland were indistinguishable, while often patient isolates from the same country were consistently different. Abaza *et al.*

(1991) was able to correlate groups of isolates sharing isoenzyme patterns with infectivity to Mongolian gerbils (*Meriones unguiculatus*), but not with severity of symptoms in the persons from whom they were isolated, nor with geographic origin of the isolates.

All of the above information leads to a variety of possible conclusions: Chiefly, it can be concluded that humoral immunity plays a role in parasite clearance and resistance to re-infection with antigenically homologous strains of parasite. The parasite may be able to overcome this protection by changing surface antigens (Nash, 1994). Certain parasite strains are more likely to elicit symptoms than others, but host factors are also involved in the elicitation of symptoms. Thus, the relationship of strain and ability to elicit CMI should be investigated to determine if a relationship can exist which might be predictive of the ability to elicit symptoms.

Delayed type hypersensitivity (DTH) has been measured in mice using the *G. muris* model of infection. Ljungstrom *et al.* (1985) determined that the DTH response to sheep erythrocytes was not modified by concurrent infection with *G. muris*. Anders *et al.* (1982) found no DTH reaction to *G. muris* antigen during infection but were able to elicit a positive response in mice pre-treated with cyclophosphamide. The relationship between *G. lamblia* and the reactions seen in the *G. muris*-mouse system, considering

the numerous possible genetic constitutions of mice strains, is uncertain. In addition, *G. muris* is non-pathogenic to mice, and therefore DTH elicitation would not be predictive of the elicitation of symptoms in this model. The DTH responses elicited by *G. lamblia* should be investigated to determine the possible role of these reactions in clearance of the parasite.

The method of footpad injection has been used by Finerty and Krehl (1976) to investigate the relationship between lethal strains of malaria and protection by pre-treatment with cyclophosphamide. Mice pre-treated with cyclophosphamide were protected from death by a lethal strain of malaria, while exhibiting a greater DTH response to the antigen. Finerty and Krehl (1977) described a system of footpad injection and antigen sensitization to determine DTH and define immunologic memory and other parameters of the DTH response. Finerty *et al.* (1978) showed a dose dependant relationship of mice immunized in the footpad with *Trypanosoma rhodesiense*.

The present study was designed to use isoenzyme banding patterns to classify strains of *G. lamblia* with respect to their DTH response. There are many enzymes which have shown polymorphism among strains of *G. lamblia*. If the DTH response can be shown to vary between strains, then there may be enzyme banding patterns which are predictive of this variation. Since the CMI response may play a role in pathology, the banding patterns of a given level of DTH may

be good indicators of the pathology induced by a strain of *G. lamblia*. Currently, although many strains of *G. lamblia* have been differentiated on the basis of their isoenzyme patterns, the specific patterns which may be related to virulence have not yet been ascertained. Determination of differential response to CMI and a relationship of this characteristic to isoenzymes may allow for prediction of virulence on the basis of isoenzymes.

Variation in CMI response between isolates of *G. lamblia* has not yet been shown, but would help to explain the range of pathogenic response demonstrated by hosts infected with different strains of *G. lamblia*. The objective of this study is to determine if there are consistent differences between groups of isolates with respect to elicitation of CMI response.

To accomplish the determination that strains have an intrinsic ability to cause a differential CMI response, an attempt to isolate clinical specimens of *G. lamblia* in axenic laboratory culture was made. Isolates were to be characterized with respect to isoenzyme banding patterns and grouped on this basis. The ability of these groups of isolates to elicit delayed type hypersensitivity reactions by foot pad inoculation were to be measured and compared to a standard laboratory strain of *G. lamblia*.

Additionally, it was planned that monoclonal antibodies would be developed to surface antigens of *G.*

*lamblia*. These antibodies would have been used to locate *G. lamblia* antigens as presented by intestinal cells of an infected Mongolian gerbils. Presentation of antigen by these cells would have supported the hypothesis that intestinal epithelial cells are targets of immune response in *G. lamblia* infection.

**Materials and methods:**

Overview:

The strategy employed in this study was to obtain *G. lamblia* from patients and to attempt excystation of these isolates by a combination of methods. Isolated strains growing in culture were to be cloned and characterized with respect to isoenzyme banding patterns. Clones and isolated strains were then to be inoculated into one hind footpad of a mouse. The opposite hind footpad would then be inoculated at a later date and swelling observed and measured over time to determine both immediate and delayed hypersensitivity. If necessary, a total of four inoculations per mouse could be given, one primary, and three followed for development of hypersensitivity response. DTH measurement was to have been accomplished by the following procedure: Albino Swiss (Ganguly *et al.*, 1985), C57B/6J (Ljungstrom *et al.*, 1985), BALB/c or C3H/He (Anders *et al.*, 1982) mice aged 4 weeks were to have been pre-tested on a small scale. If none of the mice demonstrate DTH, Mongolian gerbils were to have

been tested. All of the mice and the Mongolian gerbils can harbor *G. lamblia* infection at some point in their development. Mongolian gerbils may be better hosts as they harbor infection as adults whereas mice may only be infected as juveniles. Mice primed with cyclophosphamide two days before sensitization were to have been sensitized with a dose of 100,000 killed organisms in a 0.1 ml inoculum, and challenged one week later with a similar dose in the opposite foot. If this dose was not sufficient to bring about a DTH response a second challenge was to be administered after one week, and a third the following week if necessary. DTH was to have been measured with a digital caliper.

## Specific protocols:

### Parasites:

Most patient isolates were obtained mostly from the U.S. Naval Medical Center, National Capital Region, were about one week old upon receipt, and had been stored in plastic specimen jars in a 4°C refrigerator. Some specimens were obtained from individuals who contributed directly to the laboratory, and were generally brought in fresh (within 24 hours (h) of production).

Two commercially available strains of parasite were routinely grown in the laboratory: WB (ATCC 30957), and PI (ATCC 30888).

## *Giardia lamblia* culture:

*Giardia lamblia* was cultivated in Keister's (1983) modified TYI-S-33, with minor modifications, referred to in this paper as BI-S-33 (see appendix 1). Culture vessels used routinely were screw capped borosilicate glass tubes with a volume of about 13-15 ml dependant on the type of tube; several lots and supplies of tubes were used. Tubes were incubated at a slant of about 15° from horizontal. In this way a monolayer of trophozoites grew on one side of the tube with a cell pellet collecting in the bottom. Passage was accomplished by chilling the culture tubes in ice for ten minutes and suspending the trophozoites into a homogeneous solution by inverting the culture tube ten times. A small volume of the suspended culture (about 0.5-1 ml) was then removed from the culture tube with a sterile, cotton plugged, glass, 9 inch, pasteur pipette and inoculated into a fresh tube of medium which had been pre-warmed to 37°C.

## Isoenzymatic Characterization:

Several protocols for isoenzymatic characterization were attempted. Protocols were attempted as described with polyacrylamide, agarose, and starch gels (see appendix 2) using several buffer systems. Detailed descriptions of the formulations for enzyme detection were taken from Harris and Hopkinson (1976).

Giardia stock cultures were grown as above, chilled on an ice bath for 10 minutes, pelletted in a refrigerated centrifuge for 10 min at 4°C at 600 x g, resuspended in sterile phosphate buffered saline (PBS) (see appendix 3) and pelletted again following the above procedures, washed again 2 times, then suspended in an 0.1 mM EDTA solution. *Giardia lamblia* trophozoites were fractured either by freeze/thaw by immersing in liquid nitrogen for 10 minutes and thawing in a 37°C water bath until thawed completely, repeated for a total of three times, or by sonication at 60% power for 60 seconds with a Fisher sonic dismembrator model 300. Protein content of this solution was determined by the Coomassie Brilliant Blue G-250 method (Bradford, 1976) (BioRad) (see appendix 4).

Isoenzyme profiles attempted for the following enzymes: Malate dehydrogenase (EC 1.1.1.37; MDH), Malic enzyme (EC 1.1.1.40; ME), 6-Phosphogluconate dehydrogenase (decarboxylating; EC 1.1.1.44; PGD), Glucose-6-phosphate dehydrogenase (EC 1.1.1.49; G6PD), Hexokinase (EC 2.7.1.1; HK), Phosphoglucomutase (EC 2.7.5.1; PGM), Glucose phosphate isomerase (EC 5.3.1.9; GPI). These enzymes were chosen as a cross section of enzymes which had been used successfully for distinguishing between strains of *G. lamblia* as reported in the literature (Andrews *et al.*, 1992, Baveja *et al.*, 1986, Bertram *et al.*, 1983, Cedillo-Rivera *et al.*, 1989, Korman *et al.*, 1986, Meloni *et al.*, 1988, Meloni *et al.*,

1989, Morgan *et al.*, 1993, Moss *et al.*, 1992, and Proctor *et al.*, 1989). Additional enzymes were tried as well owing to the availability of enzyme assay protocols for these enzymes.

Sonicated *G. lamblia* was run on polyacrylamide (Laemelli, 1970) (5-15% gradient with discontinuous stacking gel), agarose, and starch gels (see appendix 2). Starch gels were most frequently used in later trials due to more reproducible results. Enzymes used for further trials were: FUM, PK, GCD, HK, ME, MPI, XDH, IDH, ARGK, HAD, AK, TPI, GR, EST, PGM, PGI, GPDH, LDH, LAP, SDH, and 6PGD using 3 buffer systems: TCss, MORPH, LiOH following general procedures of Harris and Hopkinson (1976).

## Excystation:

### *In vitro* excystation:

Fecal samples from patients with giardiosis were obtained from various sources. Because of the delay between sample collection, diagnosis and receipt by the laboratory (usually 1 week), no trophozoites were ever observed in any of the fecal samples received. Over 100 excystation attempts were made.

A good review of excystation methods which have been used by various researchers is given by Schaefer (1990). All methods share the common steps of a pre-excystation incubation in a low pH medium, which perhaps simulates

passage of the cyst through the stomach. Some the protocols include a proteinase in this incubation, while many include this as a separate step. The final step is incubation in a medium suitable for excystation and growth of trophozoites. In the laboratory nearly all combinations of these were tried. Numerous aliquots of all specimens were subjected to a variety of protocols in an effort to find one which would be effective in excystation. All of these methods are relatively similar in their requirements, and so several may be tried at once, or in close succession with minimal logistical interference. Below are outlined the specific methods used for excystation:

Cyst suspensions were used after dilution in either PBS or distilled deionized water ($dH_2O$), and/or purified. Purification procedures included: passing through a fine mesh screen, centrifugation in either $dH_2O$ or PBS (depending on the initial diluent) until the supernate was clear, centrifugation (600 x g, 10 min, 4°C) through 0.4 M sucrose and collecting the pellet, centrifugation (600 x g, 10 min, 4°C) over 0.8 M sucrose collecting the interface, or centrifugation (600 x g, 10 min, 4°C) through a 0.4 M / 0.8 M discontinuous sucrose gradient collecting the 0.4 M / 0.8 M interface. Each fraction was collected and examined to determine the presence and purity of cysts. To recover a higher yield of cysts, cyst positive fractions were further purified to improve yield. After centrifugation cysts were again washed in $dH_2O$ or PBS to remove sucrose. On several

occasions cyst preparations were passed through a G25 sephadex column (Douglas *et al.*, 1987) to remove bacteria.

Cysts suspensions purified in this manner were exposed to excystation treatment which consisted of exposure to an acidic induction medium with or without L-cysteine, exposure to a proteinase medium and final incubation in growth medium. The acidic induction medium was either a Hanks or Tyrode's balanced salt solution, or distilled water base with pH values from 2 to 5 with HCl as a proton donor. The proteinase medium consisted of the same base as the acidic induction medium but with different grades of trypsin or pepsin used in different trials (pH 2-8). Washing was performed between each incubation on some experimental runs with 0-3 washes in the base medium (Hank's, Tyrode's, $dH_2O$). Several methods were used to control bacteria, including different washing protocols and antibiotics (penicillin, streptomycin, gentamicin, piperacillin, and or amikacin) to the final medium, or semi-solid agarose (0.11% agarose in BI-S-33) medium. Growth medium used was either Keister's modified TYI-S-33, or Keister's modified BI-S-33. On several trials the medium was modified with addition of fresh bovine bile, or additional dehydrated bovine bile (see appendix 5).

A typical trial included up to six fecal samples with 4 protocols. The number of trials per fecal sample was limited by the amount of fecal material, and the freshness of the specimen. Generally all permutations of low pH

induction with or without L-cysteine, presence or absence of proteinase treatment and inoculation into growth medium with or without additional bile and with or without antibiotics. Trials were generally replicated 3 to 4 times dependant on the amount of fecal material available.

*In vivo* excystation:

Another method of excystation was *in vivo* excystation. To attempt this, four suckling mice were obtained and inoculated with cyst suspensions by gastric lavage. After a waiting period from 1 to 4 weeks the mice were sacrificed at a rate of 1 per week and their intestinal tracts examined for presence of trophozoites.

**Results**:

Enzyme electrophoresis:

Protein concentration as determined by the coomassie blue assay was 0.2 mg/ml. Good bands were seen with PGM (buffers: TCSS, LiOH, and Morph), and ME (buffers: TCSS, LiOH, and Morph) using starch gel electrophoresis. No difference was seen in the banding patterns of the clone or the parent stock of WB (ATCC 30957). Strain PI (ATCC 30888) could be differentiated from WB (ATCC 30957) on the basis of PGM staining. After repeated trials to establish any pattern on the stock cultures maintained in the laboratory,

it was impossible to establish consistent banding patterns with other enzymes tried.

Excystation:

*In vitro* excystation:

No trophozoites were ever observed in any of the fecal samples received, although over 100 attempts were made. In most preparations bacterial overgrowth was present within 4-24 h.

*In vivo* excystation:

None of the mice were found infected. The reason why the mice were killed on such a rapid schedule was that they had begun to die, because their mother failed to

produce sufficient milk. A second pregnant mouse delivered an entire litter still-borne. A replacement mouse failed to deliver a litter. Further trials were precluded due to difficulty of supply and animal care.

**Discussion:**

Elicitation of DTH using the laboratory strains WB (ATCC 30957), and PI (ATCC 30888) was not attempted. It was felt that this would not be a judicious use of animal resources. The ability of strain WB (ATCC 30957) to provoke a DTH response after pre-treatment with cyclophosphamide is

already known, and strain PI (ATCC 30888) is of feline origin and its relevance to human pathogenicity is unknown (Bertram, *et al.*, 1983).

### Enzyme electrophoresis:

It was found that starch gels produced the most effective and reproducible results. This however might be due to the specific reagents used in the particular trials made with starch gels. The multitude of reagents, especially with complex organic reagents which might contain impurities, causes the search for effective specific staining of enzymes to be highly resource intensive. It is far better to start with demonstrated protocols than to attempt to reproduce protocols from the literature.

### Excystation:

#### *In vitro* excystation:

Although *in vitro* excystation has been described in the literature, it is difficult to accomplish in the laboratory. There may have been excystation on several occasions, but the rapid growth of bacteria probably made excystation events impossible to distinguish, and untenable to sustain in culture. Better methods of purification of the cysts from the fecal bacteria should be developed to facilitate *in vitro* excystation.

*In vivo* excystation:

*In vivo* excystation has been described as a suitable alternative for *in vitro* excystation. Had the animal supply been of better quality and easier to access it might have been possible to accomplish in this laboratory. The difficulty of pairing animals of suitable age and species with supplies of cysts led to the failure of this system. The problem with *in vivo* excystation is the uncertainty of the origin of the cysts. If Mongolian gerbils are used, they may already harbor *G. lamblia* parasites which may be of a different strain than those which are to be excysted, thereby leading to unintended results. Although Mongolian gerbils can be cured of *G. lamblia* infection by the use of chemotherapeutics, Mongolian gerbils which had been previously infected may have been rendered immune and unable to serve as a suitable excystation host, or there might be only a partial cure. Specific pathogen free Mongolian gerbils are also available but were not used due to their expense. Suckling mice avoid this problem as the progenitor generation are generally unsuitable for *G. lamblia* infection, being prone to *G. muris* instead. Therefore if the suckling mouse are born in captivity in a *G. lamblia* free environment, the *G. lamblia* which they produce will be entirely from the intended source. This is however dependant on a reliable source of suckling mice.

Excystation overall:

It was the failure of excystation which led to the failure to prove the hypothesis that CMI might play a role in pathogenesis, and that different strains of *G. lamblia* are capable of eliciting different levels of DTH. The hypothesis is still a viable one for testing. The role of CMI in the pathogenesis should be investigated further. One alternative means of this investigation might be to compare the cytokine response in individuals with symptomatic versus asymptomatic giardiosis.

Using a test for CMI which is not dependant on the excystation of different strains of *G. lamblia* avoids the problems of contamination of these strains with animal borne strains, bacterial, viral, mycoplasma, or other giardial symbionts which may change the ability of a particular strain to elicit symptoms, and selection bias of the excystation method chosen. Any of these possibilities could cause anomalous results in testing of *G. lamblia* strains. And, even though cloning the strain would provide for genetically pure samples of *G. lamblia* for testing, the selection pressures of excystation and cloning increase the possibility that the clones are not representative of the population of *G. lamblia* found in patient and non-patient communities.

**Conclusions:**

The project was terminated due to the failure of excystation. The hypothesis remains untested, and still retains its significance and should be tested at a later date or through a different methodology. This was not attempted in our laboratory due to difficulties in obtaining laboratory animals of sufficient quantity and quality. Another significant problem with this study was that there was simply not enough fresh fecal material to ensure that excystation would be successful.

If excystation were successful, the prospects for success in this study are great. A laboratory with a collection of specimens which were recently isolated from a clinical setting should attempt to determine the relationship between isolates and the ability to elicit DTH or other cellular immune responses. This type of characterization would more fully characterize the isolates, may aide in the determination of the mechanism of pathogenesis, and may be valuable in monitoring the changes in the potential pathogenicity of these isolates through extensive serial sub-culture.

## Hybridoma Antibodies to *Giardia lamblia*:

### Introduction:

The objective of this study was to develop a new, rapid, reliable and inexpensive diagnostic test for giardiosis, without the need for sophisticated equipment or refrigeration to store the components. Such a test could be performed under adverse field conditions.

Several diagnostic tests for giardiosis are currently in use. Most common is the direct examination of stool (either fresh stool or formalinized) by light microscopy. Various stains (eg. Lugol's, trichrome, etc.) may be used to aid in identification. Also numerous techniques may be used to concentrate the sample (eg. 1x g sedimentation, formalin/ethyl acetate concentration, etc.). Alternatively, intestinal juices may be collected by duodenal intubation or string capsule and the material collected may be examined for the presence of trophozoites or cysts.

These methods have been replaced in some laboratories by the use of commercially available fluorescein-conjugated antibody techniques. This test suffers from the need to use fluorescence microscopy which is not universally available. Also, fluorescent slides are not permanent, therefore complicating quality control. A good quality control program may involve re-examination of positive slides by a qualified expert, days or weeks after

initial diagnosis.

All of the above-described microscopic methods suffer from the necessity of *G. lamblia* cysts or trophozoites to be present in the fecal material collected. *Giardia lamblia* cysts are not shed constantly throughout the infection, so any one or even several stool examinations may fail to detect current infection. String capsule detection may be more reliable in this regard, but may face some resistance from the patient due to the nature of this test.

Serologic tests for *G. lamblia* antibodies are available. These tests detect the presence of anti-*G. lamblia* antibodies in the serum of the host, but do not distinguish between current or past infection. Following the titer of these antibodies may be helpful in determining the course of infection, and determining the specificity of the antibodies may have some epidemiologic use, but in general, serologic diagnosis is not an effective diagnostic tool in giardiosis.

An enzyme-linked antigen capture test for giardiosis already exists; however this test by its nature is unsuitable for use in a field setting, as it requires the use of refrigeration for its components, and requires the use of glass tubes which are easily breakable and must be replaced after each sample. This test requires dilution of a large quantity of the sample in buffer which adds weight to the test, requiring the field worker to carry a sufficient amount of diluent for sample preparation of each

sample which is then transported back to the lab. Thus, treatment is delayed until the sample can be diagnosed, and a field worker can return to the field and attempt to re-locate the individual to be treated. Furthermore a positive reaction can be subtle, and may be misread by a technician.

In this study, hybridoma antibodies were to be developed which could be used in a dipstick test which would be easily field portable, easy to read, and inexpensive. This test would consist of a dipstick with a dot of antibody pre-applied to it, and a developing reagent or series of reagents. A small quantity of the sample would be applied to the dipstick. After washing, antigen bound to the antibody would be detected by a second antibody. In the final test this second antibody would be conjugated to a detection system such as microspheres containing a dye, or an enzyme which would be reacted with its substrate to produce a color change. During development the detection system would be accomplished an indirect ELISA. The proposed test would employ dipsticks which could be taken to the field. This would reduce the weight and bulk of supplies required to be transported to the field, providing rapid diagnosis.

**Materials and methods:**

The protocol used for production of hybridomas followed that of Galfre and Milstein (1982). BALB/C mice were immunized with whole *G. lamblia* ($1 \times 10^5$/ 200 µl)

injected into the tail vein. Sera from these mice were tested by indirect immunofluorescence (Mellors *et al.*, 1955a and b, and Weller and Coons, 1954) for presence of anti-giardial antibodies against whole *G. lamblia* dried on 12-well teflon coated slides (see appendix 6). When a mouse demonstrated a serum titer of about 1:200 the spleen was removed and spleen cells fused with myeloma cells, X63.Ag8.653 (Kearney *et al.*, 1979). Hybridomas were grown in HAT (hypoxanthine, aminopterin, thymidine) medium.

This medium is formulated by: gentamicin sulphate (50 mg/ml)- 1 ml, heat inactivated fetal calf serum- 200 ml, NCTC 109- 100 ml, 100 x l-glutamine (20 mM)- 10 ml, 100 x sodium pyruvate (100 mM)- 10 ml, 100 x insulin (20 units/ml)- 10 ml, 100 x oxaloacetate (13.2 mg/ml)- 10 ml, 100 x HT supplement ($1x10^{-2}$ M hypoxanthine/ $1.5x10^{-4}$ M thymidine- 10 ml, 100 X aminopterin ($4.3x10^{-5}$ M)- 10 ml, Dulbecco's minimum essential medium pH 7.2 q.s. to 1000 ml. Adjust the pH to 7.15-7.2, sterilize by filtration and store at 4°C for several weeks.

Aminopterin is fatal to unfused myeloma cells and the unfused spleen cells will die by natural senescence. After several weeks the medium containing aminopterin was replaced with HT (hypoxanthine, thymidine) medium (same as HAT, but without aminopterin). Cell culture supernate was collected and assayed for antibody production by the immunofluorescence method (see appendix 6). Cell culture supernate was also assayed against *G. lamblia* washed whole

cells in 96 well microtiter plates using horseradish peroxidase conjugated goat anti mouse IgG (H and L) (Kirkegaarde and Perry Laboratories, Gaithersburg, MD)(KPL) and developed with an appropriate 1 step ABTS (2,2'-azino-di[3-ethyl-benzthiazoline sulfonate (6)]) peroxidase substrate (KPL). Positive controls were sera from immunized mice, and negative controls were normal mouse serum (both diluted 1:160). Developed plates were incubated at room temperature for at least 15 minutes before being read. Wells were considered positive if the absorbance at 405 nm was greater than two standard deviations more than the average of all wells containing culture supernate. In all cases these wells were also at least one standard deviation greater than the average of all negative control wells.

Cells from positive wells (by both immunofluorescence or microtiter plate assay) were removed from 96 well culture plates by aspiration with a pasteur pipette and planted in wells of 24 well culture plates. These wells were observed for growth and the culture supernates were assayed again to ensure that antibody producing cells had been transferred. Positive wells from 24 well plates were transferred to 75 $cm^2$ culture flasks.

Antibodies produced in this fashion were isotyped by an ELISA kit (Pharmingen, San Diego, CA). The antibodies were further characterized with respect to the antigen to which they bound by Western blot immunoanalysis (Towbin *et al.*, 1979, and Renart *et al.*, 1979).

*Giardia lamblia* were sonicated as above and the *G. lamblia* antigen was boiled in sample buffer containing sodium thiocyanate and run on 5-15% gradient, discontinuous, polyacrylamide gels (Laemelli, 1970) along with molecular weight standards (BioRad). Gels were western blotted (Towbin *et al.*, 1979, and Renart *et al.*, 1979) to transfer the proteins to nitrocellulose paper and the nitrocellulose paper stained with amido black. Strips representing lanes of *G. lamblia* antigen were cut out and probed with the appropriate hybridoma supernate and followed by with alkaline phosphatase conjugated goat anti mouse IgG (KPL). This preparation was developed with ABTS peroxidase substrate (KPL) and the bands compared with molecular weight standards which were still stained with amido black.

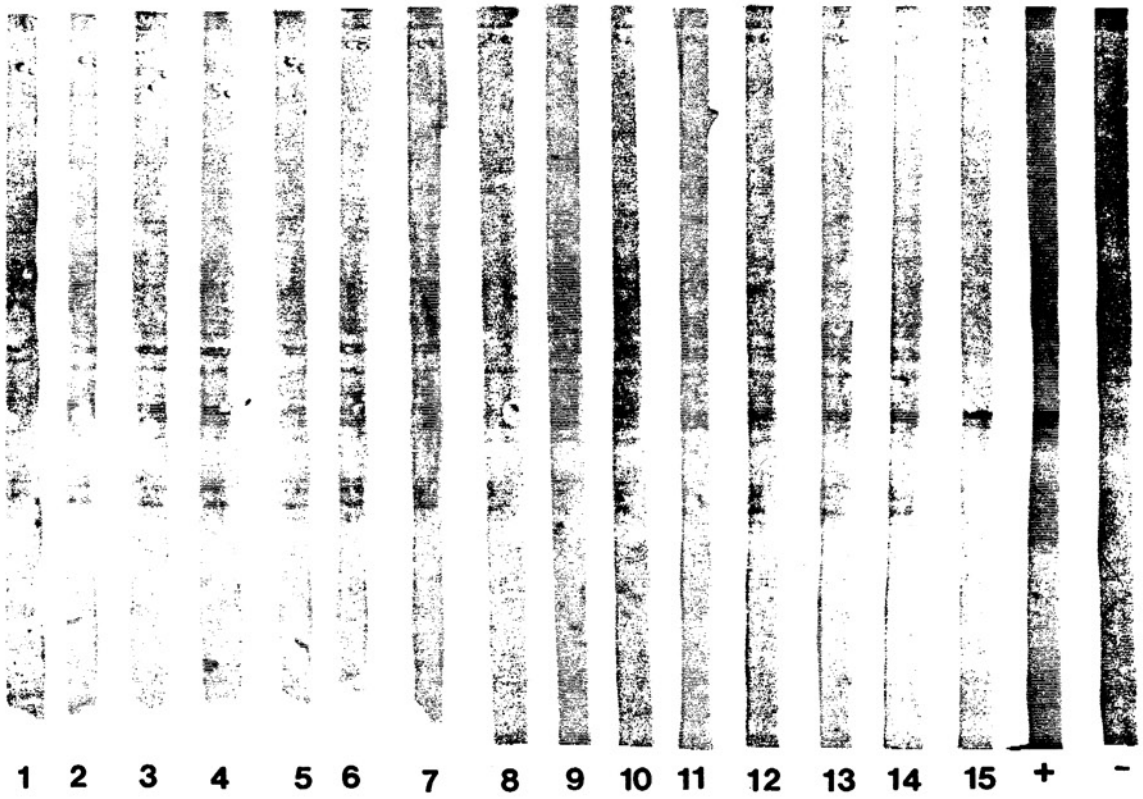
**Results:**

A hybridoma was created which produced antibodies specific for antigens of 47.8 kd. The antibodies were $IgG_1$ with kappa light chains. Other hybridomas were far less reactive against the antigens on western blot analysis. Figure 1 shows western blots of 15 hybridoma lines. The line designated R3B4 is the most positive. Positive and negative controls are also shown.

**Discussion:**

A variety of hybridoma lines producing antibodies against *G. lamblia* have been produced both to cysts

Figure 1 Western Blots of Hybridoma Supernatant.




Hybridoma lines depicted are designated 1 (R1B2), 2 (R1B3), 3 (R1B4), 4 (R1B5), 5 (R2B2), 6 (R2B3), 7 (R2B4), 8 (R2B5), 9 (R2C2), 10 (R2C3), 11 (R2C4), 12 (R2C5), 13 (R3B2), 14 (R3B3), and 15 (R3B4). The "+" designates the positive control, mouse sera reactive to *G. lamblia* in a microtiter plate assay at a titer of 1:400, used in the assay at 1:200 dilution, and a "-" designates the negative control, normal mouse serum diluted 1:200. The most strongly reactive hybridoma is 15 (R3B4).

(Erlandsen *et al.*, 1990), and to trophozoites (Nash and Aggarwal, 1986). The development of a new hybridoma line which produces antibodies to *G. lamblia* should provide for an increased ability to study the immunogenicity of *G. lamblia*. Since whole parasites were used for immunizing the mice, the protein identified as the antigen for the antibody produced is probably a surface antigen. Examination by immunofluorescence seemed to agree with the interpretation that the antigenic target of the antibody produced is located on the surface of the trophozoite.

The size of the antigen detected by this antibody fits well within the size range of variable surface antigens (vsp) reported by Nash (1994) of 35 to >200 kd. As there are probably at least 20, and as many as 185 vsp's which have reported in WB (ATCC 30957) strain *G. lamblia*. A specific antibody which binds to an epitope is therefore a useful tool to monitor the status of genetic recombination within the target trophozoite population, since the vsp's are by their nature highly variable, and a change in the expression in the vsp may indicate a change in the molecular conditions within the cell giving rise to a spontaneous shift in the vsp. A change in the vsp should not be over-interpreted. Since the conditions which give rise to alteration in the vsp genes have not been characterized, it is possible that culture conditions, unrelated to any other factors being monitored, cause the change in vsp antigen.

**Conclusions:**

Three separate attempts were made to develop hybridoma antibodies. The first attempt resulted in hybridoma cultures which were killed when the air conditioning system in the building was shut off. This resulted in overheating of the incubator in which the cultures were being grown and death of these hybridoma cultures. A second attempt was halted after bacterial contamination arose in culture plates. Attempts were made to control the contamination with a variety of antibiotics, by cryopreserving the cultures, and by washing in sterile medium and sterile PBS.

Successful development of an antibody producing hybridoma cell line was delayed by a 6 month wait for experimental animals. The antibody producing hybridoma line, once developed, was cryopreserved. This cell line should be of use to future investigators wishing to develop a dipstick test for *G. lamblia*.

**Studies in the development of drug resistance in *Giardia lamblia*:**

**Introduction:**

Drug resistance is an increasing concern in the management of giardiosis. *In vitro* resistance of fresh clinical isolates to metronidazole has been reported (McIntyre *et al.*, 1986, Majewska *et al.*, 1991), and treatment failures with metronidazole have been documented (McIntyre *et al.*, 1986). Various chemotherapeutic agents have been examined as possible replacements to the 5-nitroimidazole compounds.

Experimental induction of metronidazole resistance in strains initially susceptible to metronidazole has been reported (Meingassner *et al.*, 1978) by continuously cultivating *G. lamblia* in sub-lethal concentrations of metronidazole. Townson *et al.* (1992) improved upon this methodology by intermittently exposing *G. lamblia* to approximate $IC_{50}$ concentrations (the concentration which inhibits the growth of 50% of the exposed population) of metronidazole. Boreham *et al.* (1988) reported that metronidazole resistance was not a stable characteristic, and was lost after 22 weeks of growth in the laboratory in the absence of drug pressure. These workers suggested that metronidazole resistance was due to reduced uptake of

metronidazole from the medium. Upcroft and Upcroft (1993) in a review article suggested that there was an altered or diminished pyruvate-ferredoxin-oxidoreductase (PFOR) pathway which fails to activate metronidazole in resistant *G. lamblia* thereby causing metronidazole resistance.

Metronidazole and the PFOR Pathway:

One of the possible mechanisms of action of metronidazole is interruption of the PFOR pathway (Smith *et al.*, 1988). *Giardia lamblia* is an aerotollerant anaerobe. In order to produce energy *G. lamblia* can utilize the PFOR pathway to produce NADP. The pathway oxidizes pyruvate to acetyl-CoA by transfer of an electron to PFOR. Reduced PFOR is then oxidized by ferredoxin, which is reduced in the reaction. Ferredoxin is oxidized under the transfer of an electron catalyzed by hydrogenase to form hydrogen peroxide, water and a variety of other end products.

Metronidazole may act as an electron acceptor in this pathway. Metronidazole will accept a total of four electrons, resulting in a step-wise reduction of the $NO_2$ side chain of metronidazole to a nitro-free radical, a nitroso group, a nitroso-free radical, and finally a hydroxyamine. The free radical steps of this reduction are short lived and may be the actual mechanism of cellular killing. Another mechanism of action may involve metronidazole competitively preventing the further reduction

of pyruvate by preventing the re-oxidation of ferredoxin after it has been fully reduced.

That the PFOR pathway is involved in the metronidazole mediated killing *G. lamblia* is demonstrated by: the decreased efficiency of metronidazole in killing *G. lamblia* under conditions of increased oxygen tension, the decreased reliance on the PFOR pathway by metronidazole resistant *G. lamblia* strains, and the decreased overall concentrations of PFOR in metronidazole resistant *G. lamblia* strains.

Genetic stability of resistance mutations:

The genetic stability of strains which have been adapted to laboratory culture may be an artifact of selection pressure due to the environment generated in the culture of *G. lamblia*. For example, genetic recombination has been found in strains of *G. lamblia* which have developed resistance to metronidazole (Upcroft and Upcroft, 1993) and yet metronidazole resistance is lost after an extended period of growth in the absence of selection pressure (Boreham *et al.*, 1988). Under a clonal replication theory, this would signify that the pressure of drug resistance gives rise to conditions which favor mutant *G. lamblia* which increase in prominence within the colony. Removal of the selection pressure of drug exposure would then allow either a back mutation, or re-establishment of the wild type strain from the few progeny which may have survived drug exposure.

Several metronidazole resistant strains apparently have a similar genetic re-arrangement, which may be inferred to confer resistance to strains of *G. lamblia* (Upcroft and Upcroft, 1993). If this genetic recombination for drug resistance can arise in disparate stock cultures of *G. lamblia* it may be possible that the very act of culturing this organism may give rise to recombinations which select for a single or a small subset of populations.

Since no sexual reproduction has been observed with *G. lamblia* and asexual fission is known, it is likely that *G. lamblia*'s heritable traits are passed on clonally. This means that a mutation which arises will be passed to progeny and ultimately the proportion of the population with the mutation in question is related to the adaptive fitness of the mutation. Mutations which allow a clone to be better suited to their environment will be represented in an increasing proportion of the population as they flourish and produce more progeny than the less adaptively fit clones. Thus any infection, even by a single organism, may give rise to a population with several genotypes, and possibly several phenotypes if adaptive mutation is a frequent event. Le Blancq *et al.* (1991) demonstrated that genetic rearrangement events might occur as frequently as 3% per division demonstrating a great amount of genetic plasticity and a high probability of adaptive mutation.

The difficulty in determining the karyotype of *G. lamblia* may be indicative of a high degree of genetic

plasticity. In numerous attempts to determine the genotype of *G. lamblia* strains, anywhere from 3 to 9 chromosomes have been identified. Some of this discrepancy may be due to the variety of methods that have been used in karyotyping. Sarafis and Isaac-Renton (1993) found 4-6 chromosomes in several isolates using a four day contour clamped homogeneous field pulsed field gel electrophoresis, whereas Korman *et al.* (1992) found up to 9 chromosomes, and perhaps more, using a standard pulsed field gel electrophoresis. Still, while using the same karyotyping methodology with different strains, genetic polymorphism has been found between several isolates.

Albendazole for giardiosis:

In a search to replace metronidazole, numerous compounds were tested. Of the compounds tested, the anthelminthic benzimidazoles (albendazole, mebendazole, ornidazole, and thiabendazole, etc.) have shown promise by having antigiardial activity and few side effects (Coulaud and Rossignol, 1984, and Rossignol and Maisonneuve, 1983). Edlind *et al.* (1990) reported on the activity of benzimidazoles against *G. lamblia in vitro* finding that albendazole had the greatest anti-giardial activity. Albendazole has been reported as a possible chemotherapeutic agent for giardiosis on the basis of both *in vitro* (Meloni, *et al.*, 1990) and *in vivo*, field testing (Hall and Nahar, 1993).

Hall and Nahar (1993) reported a high cure rate of children in Bangladesh using albendazole for 7 days. Treatment failures were reported by Kollaritsch *et al.* (1993) after 3 days treatment with albendazole in individuals with giardiosis contracted abroad who sought treatment upon return to Europe. Other testing has shown albendazole to be ineffective in treatment of giardiosis (Kollaritsch *et al.*, 1993). Hall and Nahar (1993) showed a dose response relationship between albendazole and percent of children in Bangladesh cured of giardiosis. Their report suggests that an 800 mg dose, given singly or given for several days was better than the 400 mg dose frequently used in studies, or used in treatment of other diseases.

Chavez *et al.* (1992) demonstrated that *G. lamblia* did not develop resistance to albendazole by attempting to sub-culture *G. lamblia* cultures which had been exposed to albendazole. Chavez *et al.* (1992) showed that 1 µM concentrations were effective in killing cultures of *G. lamblia*. This fits well with the determination of physiological concentration of albendazole and albendazole-sulfoxide (the main metabolite of albendazole) as 600-1,000 ng/ml in blood, and 200-1000 ng/ml in bile (1,000 ng/ml ≈ 3.8 µM, 200 ng/ml ≈ 0.75 µM) (Saimot *et al.*, 1983).

There are several possible explanations for the poor efficacy of albendazole reported by Kollaritsch *et al.* (1993). Among these is the possibility that the *G. lamblia* was not exposed to a concentration of albendazole which was

curative for a sufficient time. This may have been due either to the route of administration, to the form of the drug, or to other host factors. Another explanation is that the parasite has developed resistance to albendazole, despite the findings of Chavez *et al.* (1992).

Hypermotility may be have been responsible for reduced drug concentration in the intestinal fluid of patients of the Kollaritsch *et al.* (1993) study whereas it may not have been a factor in the study of Hall and Nahar (1993). Alternatively the 3 day course of administration may have been too short to effect a cure, or the dosage may have been too low. The study of Kollaritsch *et al.* (1993) which found albendazole ineffectual in the treatment of *G. lamblia*, was probably a result of a low dosage of albendazole, as demonstrated by Hall and Nahar (1993).

Several authors are optimistic about the possibility of using albendazole for giardiosis (Jarroll, 1994, Hall and Nahar, 1994, Morgan *et al.* 1994, and Reynoldson, 1994, and Reynoldson *et al.* 1994). Another recent publication reports the possibility of a recently isolated *G. lamblia* strain showing decreased *in vitro* sensitivity to albendazole (Kortbeek *et al.*, 1994). The authors of this paper still felt, however, that albendazole was a "promising" potential therapeutic drug, noting unspecified differences between the resistant *G. lamblia* isolate and other susceptible isolates.

In light of the growing recognition that albendazole is a candidate to become an alternative to metronidazole for treatment of giardiosis, it is necessary to determine if albendazole resistance is likely to occur. And, if drug resistance does occur in nature the possible mechanisms of albendazole resistance should be determined.

In the present study, albendazole resistance in *G. lamblia* was induced in the laboratory. Albendazole resistant *G. lamblia* were to be characterized with respect to several factors: stability of resistance when cultivated in the absence of drug pressure, the ability of resistant *G. lamblia* to inactivate albendazole, the ability of resistant *G. lamblia* to encyst, the existence of cross resistance to metronidazole, the effect of growth in prolonged exposure to albendazole, the ultrastructural differences in albendazole resistant and susceptible strains when examined by transmission electron microscopy, and the uptake of $^{14}C$-labeled albendazole from medium compared between resistant and susceptible strains of the parasite.

**Materials and Methods:**

Growth and Cloning of *Giardia lamblia*:

A laboratory strain of *G. lamblia*, WB (ATCC 30957) was cloned by the method of Gillin and Diamond (1980), in BI-S-33 containing 0.82% agarose with an inoculum calculated to contain 3 trophozoites. Clones which grew into colonies

were inoculated into BI-S-33 (without agarose) grown to confluence and re-cloned using this same method. The single successfully derived clone was designated C2B. Both the strain and the clone were serially sub-cultured twice per week in Keister's modified BI-S-33 in screw-capped, borosilicate glass tubes resting at a slant in a 37°C incubator.

Induction of Drug Resistance:

Albendazole (Smith Kline Beecham/ Animal Health, Philadelphia) was prepared as a stock solution of 20 mg/ml albendazole dissolved in dimethyl sulfoxide (DMSO). When cultures had grown to confluence, 2 ml of drug diluted to appropriate concentration in BI-S-33 and filter sterilized through a 0.22 µm Millipore filter was added to each tube and each tube was inverted to distribute the drug throughout the medium. Both the parent strain and the clone were exposed to 0.23 µM albendazole for 24 h, the approximate $IC_{95}$ (the concentration which will inhibit 95 percent of the growth of the population). After exposure cultures were chilled in ice for 10 minutes, centrifuged (600 x g, 10 min, 5°C) and the old medium was poured off. Chilled phosphate buffered saline (PBS) was added to each tube and the trophozoites were resuspended by vortexing and inverting the tubes. Trophozoites were again sedimented as above and the PBS poured off. Parasites were washed this way in PBS three times. Fresh, pre-warmed BI-S-33 was then

added to the parasite pellet and the culture tubes were inverted to re-suspend the trophozoites. The trophozoites were allowed to propagate in a 36°C incubator for 72 h. Cultures were then chilled on ice for 10 minutes and inverted to suspend the trophozoites. A 0.5 ml aliquot was then inoculated into 9.5 ml fresh BI-S-33.

The treated cultures were periodically re-exposed to the approximate $IC_{50}$ once per week, depending on the growth and recovery of the exposed cultures. Duplicates or triplicates of treatment cultures were propagated. The second dose of drug was 0.12 µM; thereafter some tubes were inoculated with a higher concentration of drug empirically determined. When a tube showed viability at a higher drug concentration, that tube was passed into two tubes and the higher concentration was used in one tube and an even higher concentration of drug was used in the second tube.

Control cultures were grown in parallel to the drug exposed cultures. The control cultures were exposed to DMSO at the same concentration as in the drug on the same schedule as the drug exposure. In no case was the volume of DMSO greater than 0.5% of the final volume of medium.

Initially the sodium 3,3'-[1[(phenylamino)carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro benzene sulfonic acid hydrate (XTT) soluble formazan technique (Wright *et al.*, 1992) using phenazine methosulfate (PMS) as an electron coupling agent (Scudiero *et al.*, 1988) was used to attempt to determine viability. This technique was used to

determine viability when testing for metronidazole resistance. The test of viability in albendazole resistance was the ability to be sub-cultured.

Studies were then undertaken with the resistant populations to determine: stability of resistance, encystation ability, drug inactivation, cross resistance to metronidazole, and the effect of constant drug exposure. When the results of these studies were known, two further studies: examination of alterations in the ultrastructure of resistant and susceptible trophozoites when exposed to albendazole, and documentation of $^{14}C$-labeled albendazole uptake by resistant and susceptible trophozoites.

## Stability of Resistance:

Stability of resistance was tested by serially cultivating the organisms which had demonstrated resistance to 4.2 μM albendazole in medium without further exposure to albendazole as compared to controls to determine if resistant parasites reverted to susceptibility when grown without drug selection. Later trials with strain WB (ATCC 30957) trophozoites resistant to a 24 h exposure to 9μM albendazole showed that these trophozoites had a significant reduction in their resistance after only 2 weeks. After 2 weeks without drug exposure these strains were resistant to only 7 μM for 24 h. A 9 μM 24 h exposure rendered all of these trophozoites non-viable. After the 2 week period if exposed to 7 μM for 24 h and allowed to recover for 1 week

(medium replaced after 24 h, and cells passed once after 4 days) the cultures were again resistant to an 8 or 9 µM, 24 h exposure.

Drug Inactivation:

Resistant parasites were exposed to 4.2 µM albendazole, which showed little effect on their growth. Control cultures were exposed to the same concentrations of DMSO without albendazole. At the end of a 24 h exposure the medium was removed and filter sterilized through a 0.22 µm filter attached to a 10 ml syringe to remove any trophozoites. The medium containing drug was added to control tubes while medium from the DMSO controls was added to the resistant parasites as a control for the effect of re-utilizing medium. Tubes were observed after 24 h incubation at 37°C. They were then chilled and sedimented and the medium removed and replaced with fresh culture medium to determine if cultures were viable after further incubation. Tubes were observed for viability repeatedly for at least 72 h.

Encystation Ability:

Encystation was accomplished by the methods of Gillin *et al.* (1989). Briefly, the cultures to be encysted were pre-incubated in BI-S-33 without bile (pH 7.0) until a confluent monolayer was formed. Tubes were inverted 3X and

the medium poured off with non-adherent trophozoites. Fresh BI-S-33 supplemented with 0.5 mg/ml porcine bile, and 5 mM lactic acid, pH 7.8 was added to the culture tubes. These cultures were incubated for 66 h then chilled, centrifuged at 600 x g for 10 min at 4°C and the medium decanted. Cysts were resuspended in distilled water, stored at 4°C and photographed.

Cross Resistance to Metronidazole:

Cross resistance to metronidazole was tested by the XTT assay (Wright *et al.*, 1992) as described above, which has been used to determine the efficacy of metronidazole. The procedure used was modified from Wright *et al.* (1992) using 5 mM PMS as an electron acceptor (Scudiero *et al.*, 1988). On each plate BI-S-33 was innoculated into 8 rows. Drug was serially diluted into these rows, there were 4 rows with serial dilutions of metronidazole and 4 rows of control wells with $dH_2O$ added in the same volume as the metronidazole. Albendazole resistant parasites were planted in 2 metronidazole and 2 control rows, albendazole susceptible trophozoites were planted in the remaining rows.

Microtiter plates were prepared with appropriate serial dilutions of metronidazole (Searle, Skokie, IL), and inoculated with about $10^5$ trophozoites/well for a final volume of 300 µl/well. Plates were flooded with $N_2$ gas for 5 min at 5 PSI, sealed and incubated at 37°C for 24 h.

After 24 h 250 µl of the supernatant medium was removed, PBS (pH7.2 with 1 mg/ml glucose) was added (250 µl/well) for 1 h at 37°C, removed and replaced with 100 µl XTT solution/well (XTT 0.2 mg/ml, PMS 25 µM in PBS pH 7.2 and 1 mg/ml glucose), flooded with $N_2$ gas as above, and incubated at 37°C for 4 h. At the end of the incubation absorption was determined at 450 nm and results plotted as average absorbance versus drug concentration.

Continuous Exposure to Albendazole:

Growth of drug resistant *G. lamblia* under conditions of constant drug exposure was evaluated by incubating resistant *G. lamblia* into medium containing 4.2 µM albendazole. These cultures were incubated at 37°C and observed every 24 h for 72 h.

Ultrastructural Examination:

Cultures of resistant (9 µM, 24 h albendazole) and susceptible parasites were exposed to 9µM, 24 h albendazole, and a control, susceptible culture, unexposed to albendazole were chilled centrifuged as above and the supernatant removed. The cell pellets from these cultures contained about 3.5 $x10^7$ trophozoites and were used in the following procedure.

The protocol used was that of Chavez *et al.* (1992), with a minor modification, that the trophozoites, fixed in 0.2 M glutaraldehyde in sodium cacodylate overnight were

first concentrated on a millipore filter (0.45 μm pore, 13 mm diameter) (Taylor and Godfrey, 1970). This modification was instituted due to the inability to maintain a cohesive pellet throughout the fixation process.

The filter with parasites adhered to it was then rinsed in 1% sodium cacodylate buffer (2x 15 min), stained with 1% osmium tetroxide in 1% sodium cacodylate buffer (1 h), rinsed again in 1% sodium cacodylate buffer (2x 15 min) dried in ethanol (70% 1x 15 min, 80% 1x 15 min, 95% 2x 15 min, and 100% 2x 15 min), permeated with propylene oxide (2 x 15 min). After the first treatment with propylene oxide, the membrane filter dissolved and the pellet of trophozoites was collected in microcapillary tubes and pelletted on a hematocrit centrifuge, the pellets were collected resuspended in propylene oxide and re-pelleted until only one large pellet remained per sample. These pellets were then incubated in 50% Epon 812/ 50% propylene oxide (2x 15 min) and embedded in blocks of Epon 812.

Blocks containing cell pellets were sectioned, mounted on copper screens and some were stained with uranyl acetate and lead citrate. They were examined and photographed using an electron microscope in the Biological Instrumentation Center of the Uniformed Services University of the Health Sciences.

## Radiolabeled Albendazole Uptake:

Albendazole labeled with $^{14}C$ to a level of 1.125 mCi/g (Smith Kline Beecham/ Animal Health, Philadelphia) was made into a stock solution of 20 mg/ml DMSO. *Giardia lamblia* strain WB (ATCC 30957) resistant to 9 µM albendazole and susceptible to the same concentration were cultured. Known quantities of trophozoites were inoculated into tubes containing 9.5 ml BI-S-33, and $^{14}C$ labeled albendazole was diluted in 0.5 ml BI-S-33 to yield a final concentration of 9µM in 10.5 ml. Cultures thus labeled were incubated at 37°C for time intervals from 1 to 96 h. The first trial measured time intervals of 1, 2, 3, 18, and 38 h. After the first trial it was determined that there was no difference in uptake during the 1, 2, and 3 h time periods, but there was a difference in the later time periods. Time periods were selected for a second run as 1, 18, 24, 38, 72, and 96 h to assure that the effect seen at 38 h in the first run was completely documented. Three tubes per strain (resistant and susceptible) were used at each time period.

At the specified time interval, cultures were removed from the incubator, chilled for 10 min in an ice bath and centrifuged as described above. To determine the amount of radiolabeled drug taken up by the trophozoites, 1 ml of the supernatant medium was removed and placed in a scintillation vial. Ten ml scintillation cocktail (Aquasol 2) was added to the supernate. The rest of the medium in

each culture tube was discarded. The pellet volume (including about 1 drop of the supernate which remained after medium was discarded) was measured. These pellets had a consistent volume of 0.1 ml. The pellet was then suspended in 10 ml of scintillation cocktail (Aquasol 2), and poured into scintillation vials. Radioactive decays were counted in a TM Analytic Mark V Series scintillation counter, which compared the number of counts per minute with an external standard to determine quenching and output corrected values as decays per minute in the β-range. These values were then converted into nmol for reporting. Values were compared using the SAS statistical analysis system.

**Results:**

Induction and Stability of Resistance:

At the end of this study *G. lamblia* trophozoites were being successfully grown with repeated 24 h exposures to 9μM albendazole, and increase of 3.7 fold the greatest minimum lethal dose published (2.4 μM minimum lethal concentration after 24 h exposure) (Meloni *et al.*, 1990). XTT testing for viability could not distinguish between resistant and susceptible *G. lamblia* trophozoites (see figure 2). Possibly trophozoites which had been rendered non-viable (unable to replicate) may have retained their ability to reduce XTT to its soluble formazan end product. Finally, the ability to replicate in culture was taken as

the criterion for viability. In practice, the presence of motile trophozoites with typical morphology was used as an indicator of viability (see figure 3).

The left photograph of figure 3 shows *G. lamblia* resistant to 9 µM after exposure to 9 µM albendazole for 24 h. Many trophozoites with typical pear shaped morphology are evident in this photograph. The right photograph of figure 3 shows trophozoites of *G. lamblia* control cultures after exposure to 9 µM albendazole for 24 h. Trophozoites in this photograph are irregularly shaped and rounded.

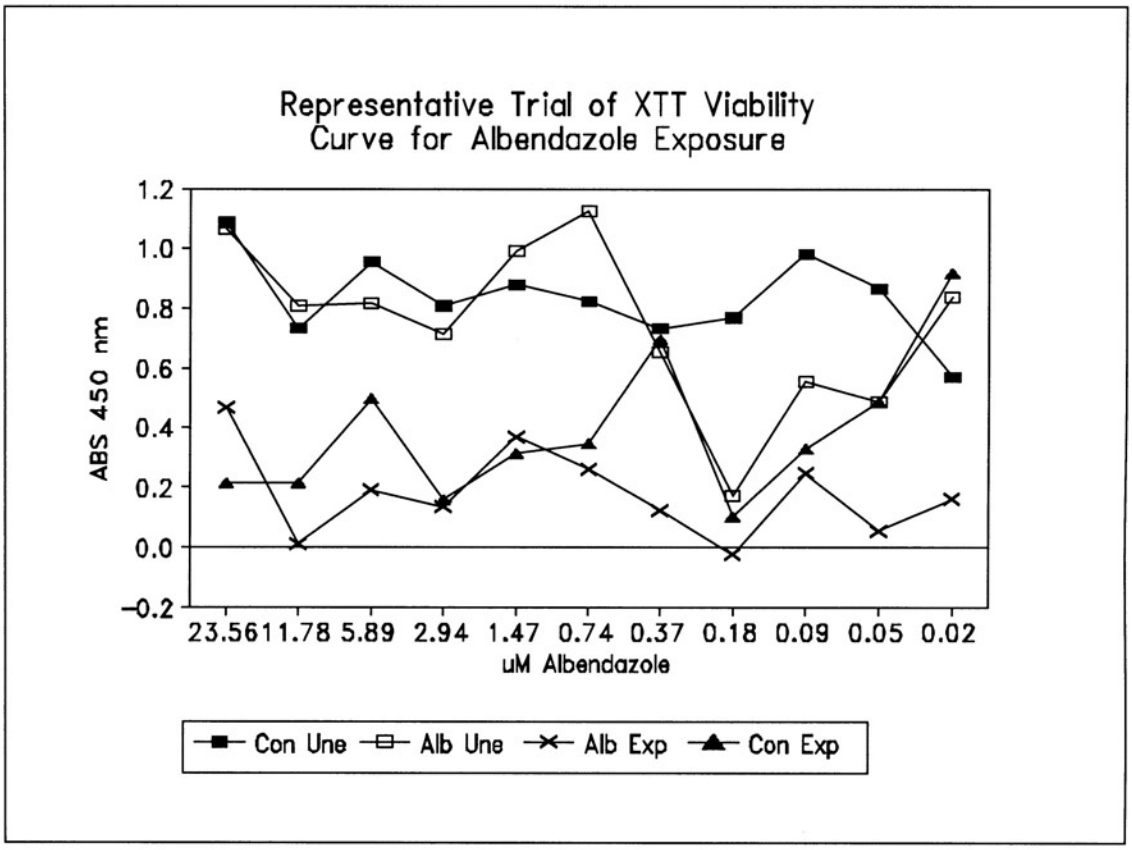
Stability of Resistance:

Resistant cultures that had been resistant to 5 µM albendazole were killed on exposure to this dosage after 10 weeks without cyclical exposure to albendazole. Trophozoites resistant to 9 µM albendazole exposure for 24 h, serially sub-cultivated without re-exposure to albendazole for two weeks were resistant to 7 µM doses of albendazole for 24 h, but were killed by exposure to 9 µM albendazole for 24 h.

Drug Inactivation:

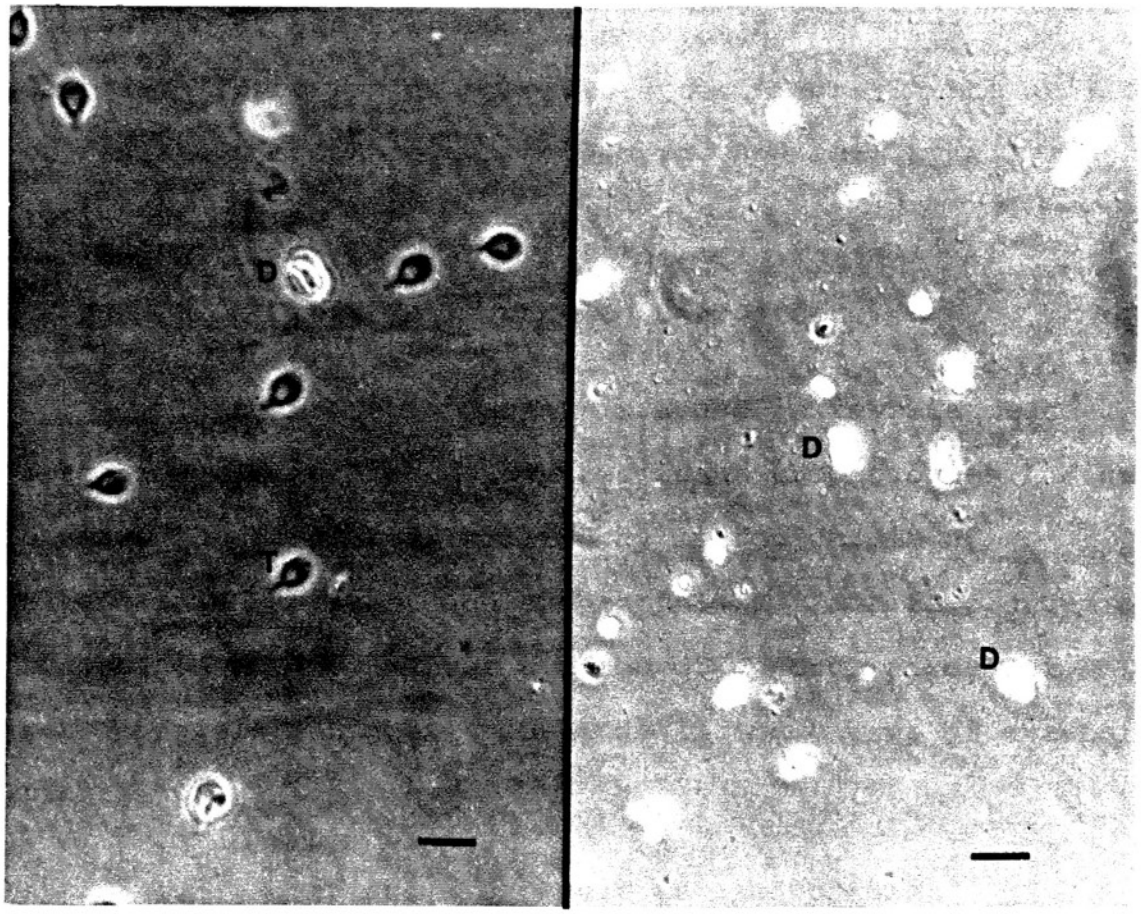
After 24 h exposure to the drug which had been pre-incubated with resistant trophozoites, all susceptible trophozoites were rendered non-viable. Growth in culture medium for 72 h failed to demonstrate any trophozoites which had survived the exposure. Resistant trophozoites incubated

Figure 2 XTT Viability Testing of Albendazole Exposure.




This graph depicts the absorbance at 450 nm (Y axis) of wells of *G. lamblia* either resistant (Alb) or susceptible (Con) to 4.5 µM albendazole, exposed to various concentrations of albendazole (Exp) or $dH_2O$ (Une) (X axis). Viability determined by XTT method, higher absorbance indicates greater viability. Data not significantly different.

Figure 3 Photomicrographs of Resistant and Control Cultures Exposed to Albendazole.




The photograph on the left depicts a culture of *G. lamblia* resistant to 9µM albendazole, after 24 h exposure to 9 µM albendazole. Note some normal trophozoites (T) and some drug affected trophozoites (D). The photograph on the right is of from an albendazole susceptible culture taken after 24 h exposure to 9µM albendazole, note numerous drug affected trophozoites (D). Bar = 10 µm.

in medium from control cultures grew and were able to be propagated normally.

Encystation Ability:

Encystation was successful. Figure 4 is a photograph of encysted resistant strain parasites of *G. lamblia*. There was no observable difference in the ability of resistant trophozoites to encyst, and form type I water resistant cysts.

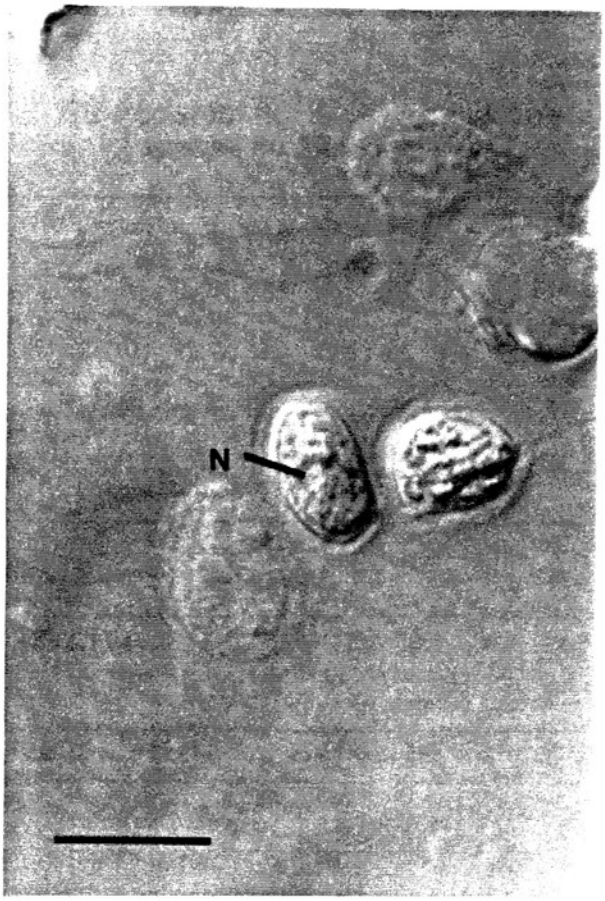
Cross Resistance to Metronidazole:

There was no apparent increase in resistance to metronidazole in albendazole resistant trophozoites. Figure 5 shows the results of killing by metronidazole of albendazole resistant and susceptible *G. lamblia* plotted as absorbance at 450 nm over a range of concentrations of metronidazole. Higher absorbance is indicative of greater viability.

Continuous Exposure to Albendazole:

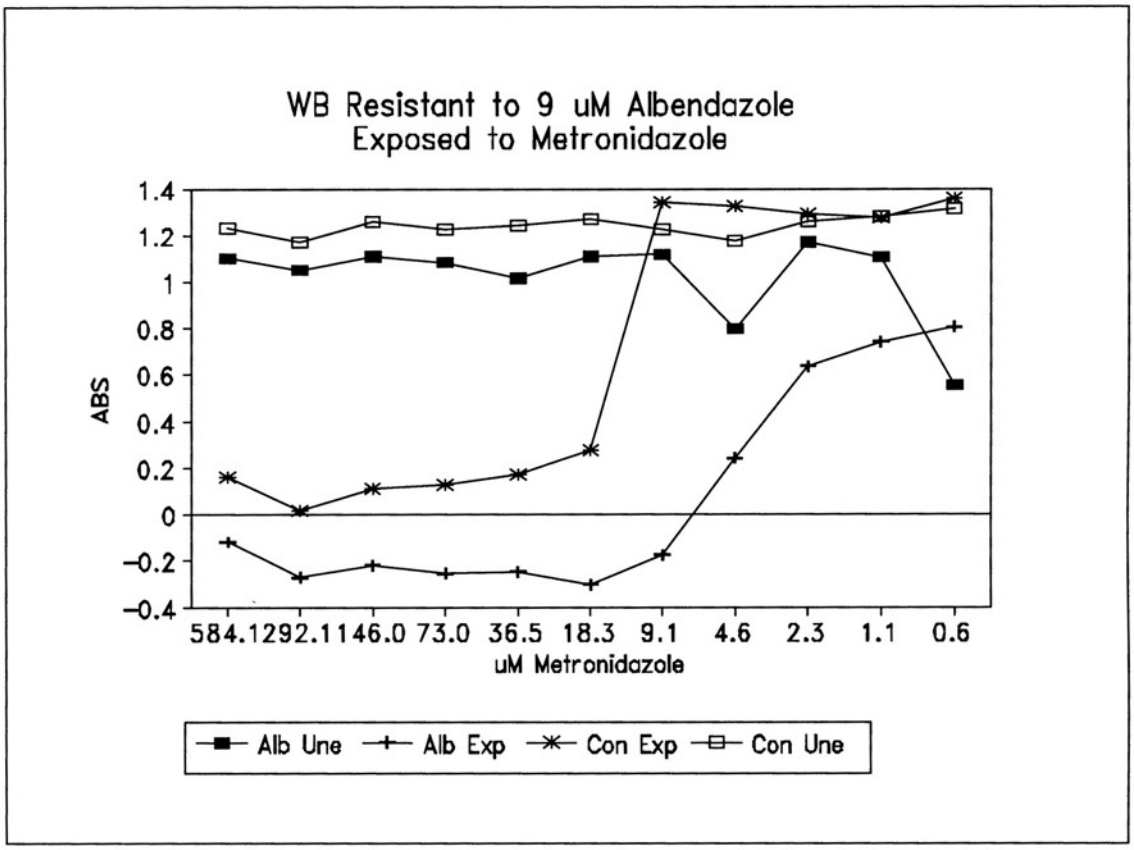
After 48 h continuous exposure to 4.2 µM albendazole. *Giardia lamblia* which were resistant to 24 h exposure was viable after 48 h exposure, and there were no motile trophozoites with characteristic shape after 96 h.

Figure 4 Photomicrograph of Encysted Albendazole Resistant *G. lamblia*.




This photo taken under Nomarski DIC demonstrates a type I *G. lamblia* cyst. It was derived from a culture of *G. lamblia* resistant to a 24 h exposure to 9 μM albendazole. N denotes the nucleus. Bar is 10 μm.

Figure 5 Killing of Albendazole Resistant and Control Susceptible *G. lamblia* by Metronidazole.




Resistant (Alb) and susceptible (Con) trophozoites exposed to various dilutions of metronidazole (Exp) or equal dilutions of $dH_2O$ (Une) for 24 h. Absorbance at 450 nm (Y axis) versus decreasing concentration of 24 hour exposure to varying concentrations of metronidazole (X axis) or a $dH_2O$. Viability determined by the XTT method, higher absorbance represents greater viability.

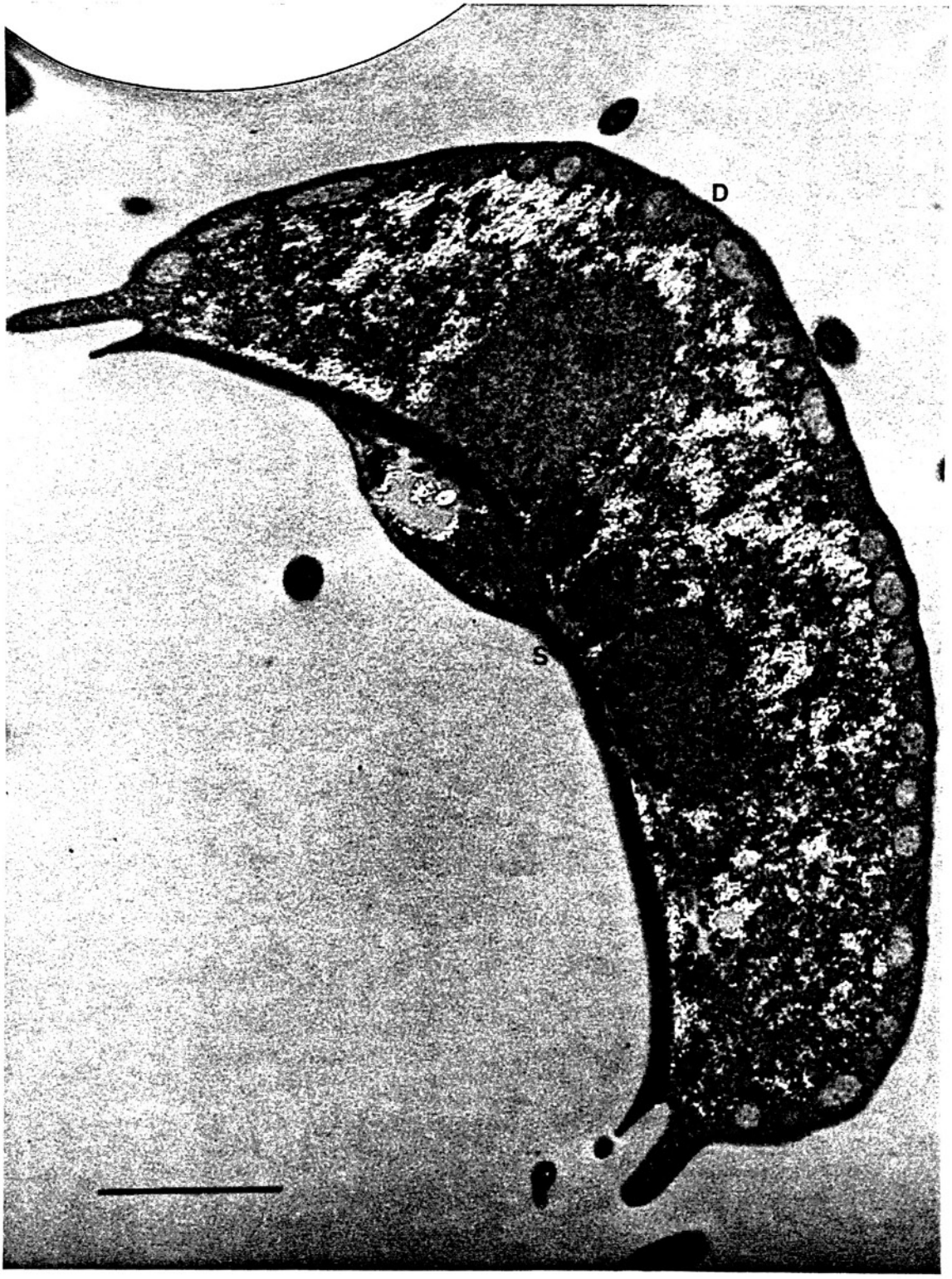
Ultrastructural Examination:

Photographs taken using electron microscopy are shown in figure 6. Both resistant and susceptible populations of *G. lamblia* showed both the typical ultrastructural damage to the striated disk, as well as examples where damage to the striated disk was less evident. Photographs 1 and 2 show control strain WB (ATCC 30957) trophozoites. In photograph 2 the funis, an organizing center for microtubules, and median body, comprised of microtubules, are visible. Photograph 3 is a higher magnification of the trophozoite in photograph 2 of the median body demonstrating the large number of microtubules in this structure. The funis is also evident in this view. Photograph 4 is the striated disk of the trophozoite in photograph 2, which shows the disk composed of tubules and associated protein ribbons. Photographs 5 and 6 demonstrate typical damage to susceptible trophozoites exposed to 9 μM albendazole for 24 h. In photograph 5, the striated disk is nearly completely degenerated. Photograph 6 shows a trophozoite which has a damaged striated disk, the funis and median body are evident in this photograph. Photograph 7 is a close up of the median body from a susceptible trophozoite after albendazole exposure, photograph 8 shows an intact funis from a susceptible trophozoite after albendazole exposure, and photograph 9 is the striated disk of a susceptible trophozoite showing typical damage to the striated disk of a trophozoite exposed to albendazole.

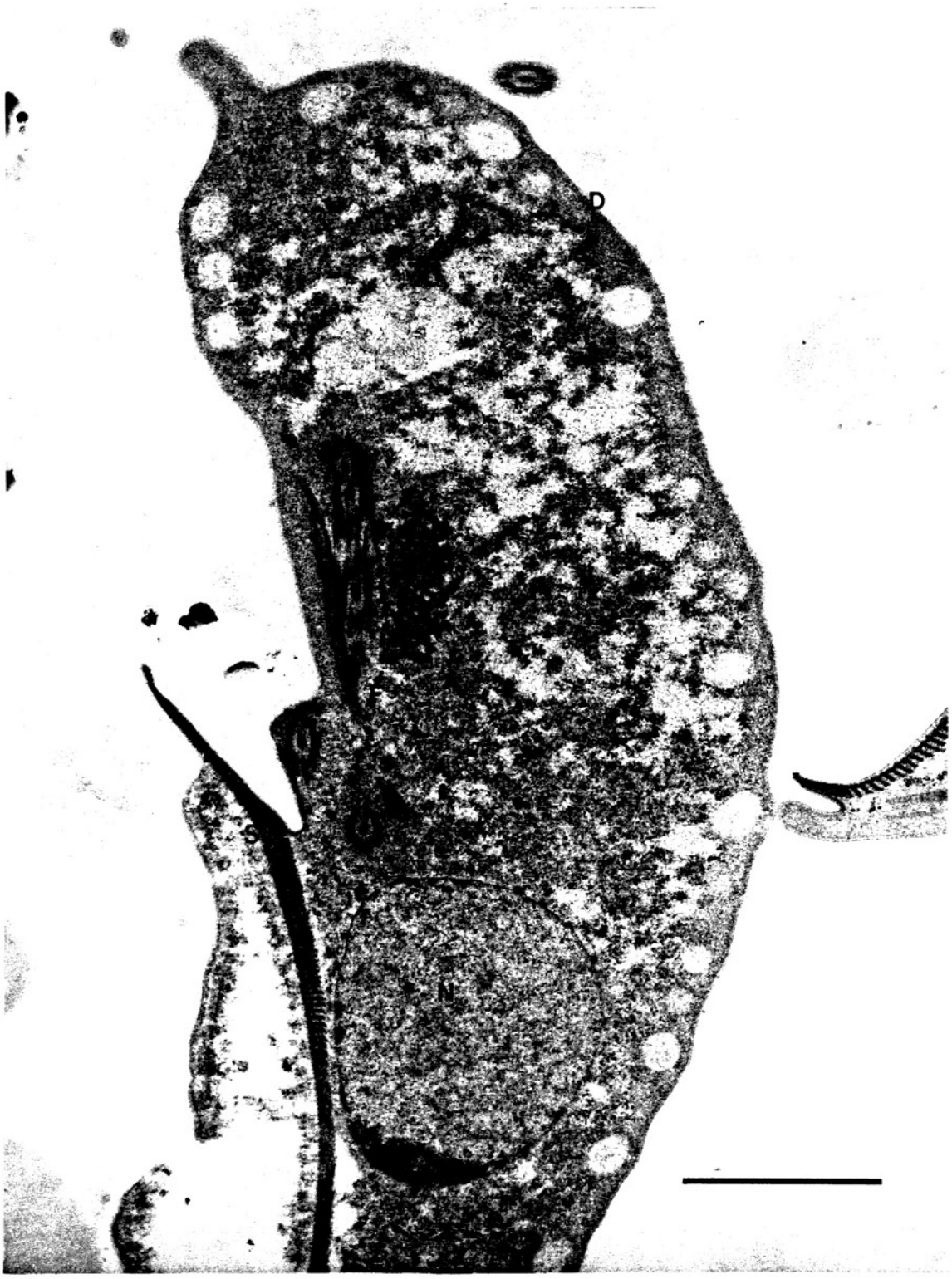
Figure 6 Electron Photomicrographs of *G. lamblia* Trophozoites Exposed to 9μM Albendazole for 24 Hours, and Unexposed Controls

The following photographs are of control *G. lamblia* trophozoites, unexposed to albendazole, susceptible trophozoites exposed to 9 μM albendazole for 24 h., and albendazole resistant trophozoites exposed to 9 μM albendazole for 24 h. Key: N= nucleus, S= striated disk, D= dorsal surface, F= funis, M= Median body.

Photograph 1: Control strain WB (ATCC 30957) trophozoite, unexposed to albendazole. Bar = 1 $\mu$m.

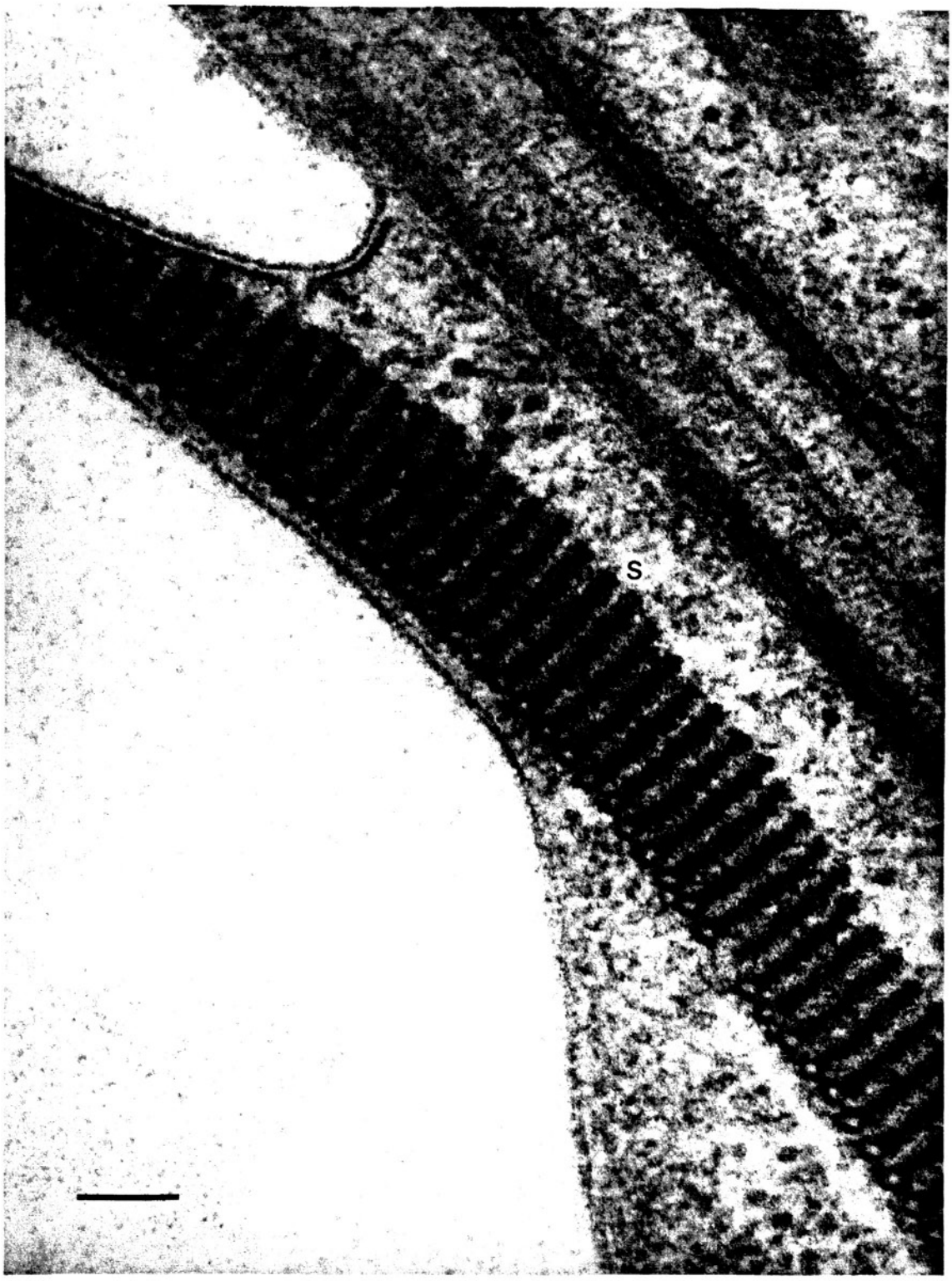


Photograph 2: Control strain WB (ATCC 30957) trophozoite unexposed to albendazole. Bar = 1 $\mu$m.

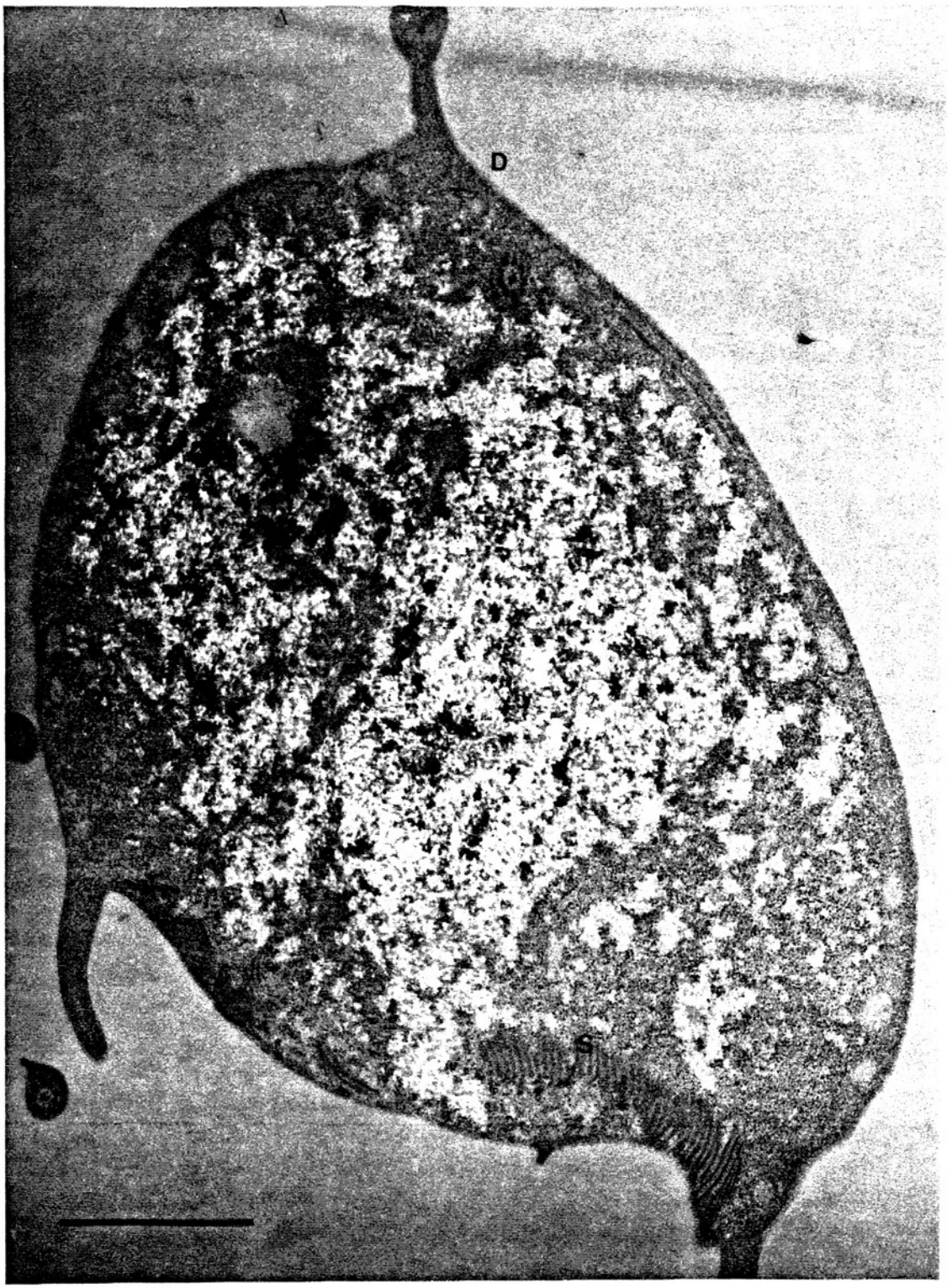


Photograph 3: Enlargement of median body and funis from photograph 2. Bar = 0.1 $\mu$m.

Photograph 4: Enlargement of striated disk from photograph 2. Bar = 0.1 $\mu$m.

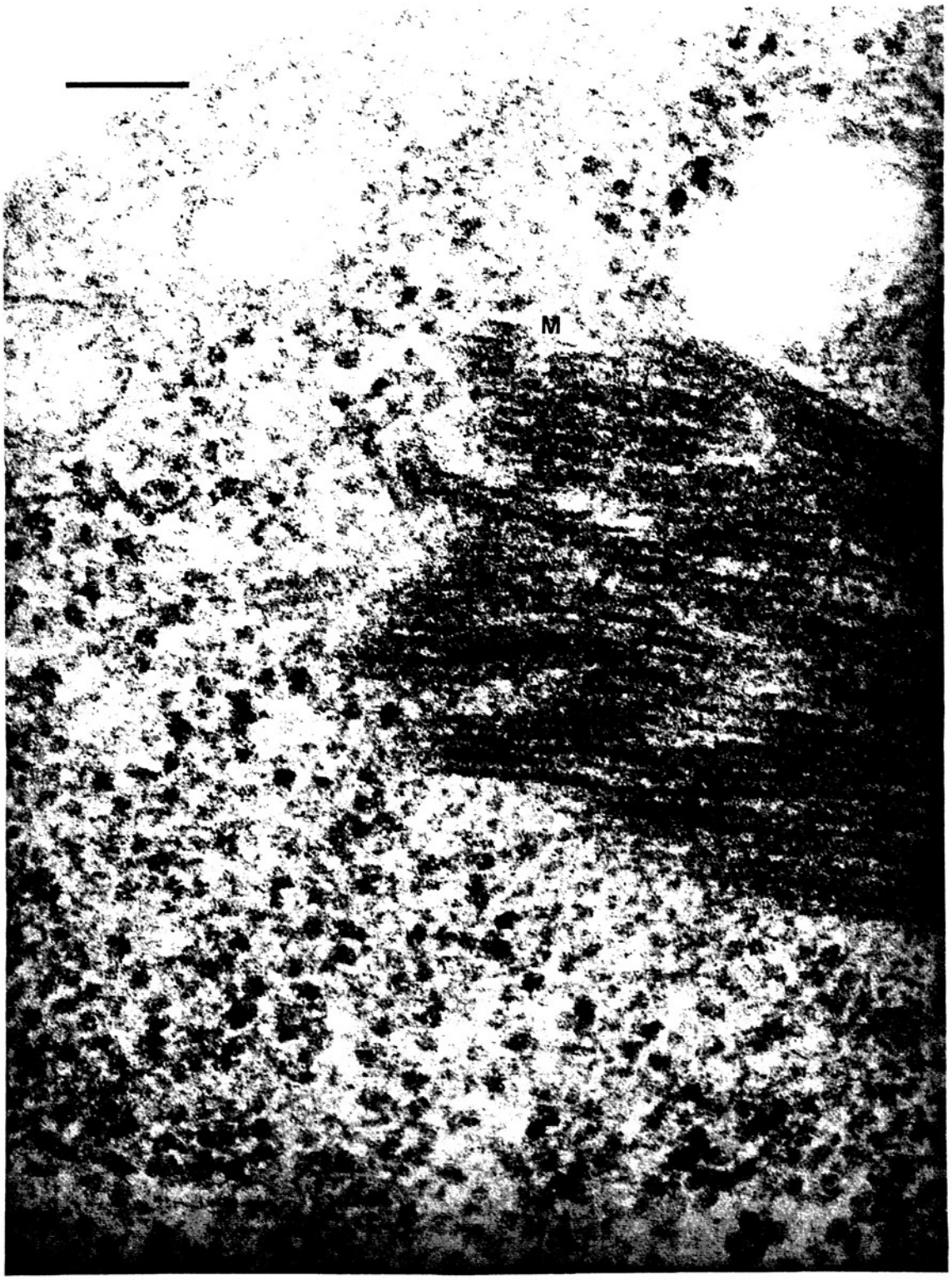


Photograph 5: Susceptible trophozoite exposed to 9 μM albendazole for 24 h. Note damaged striated disk. Bar = 1 μm.

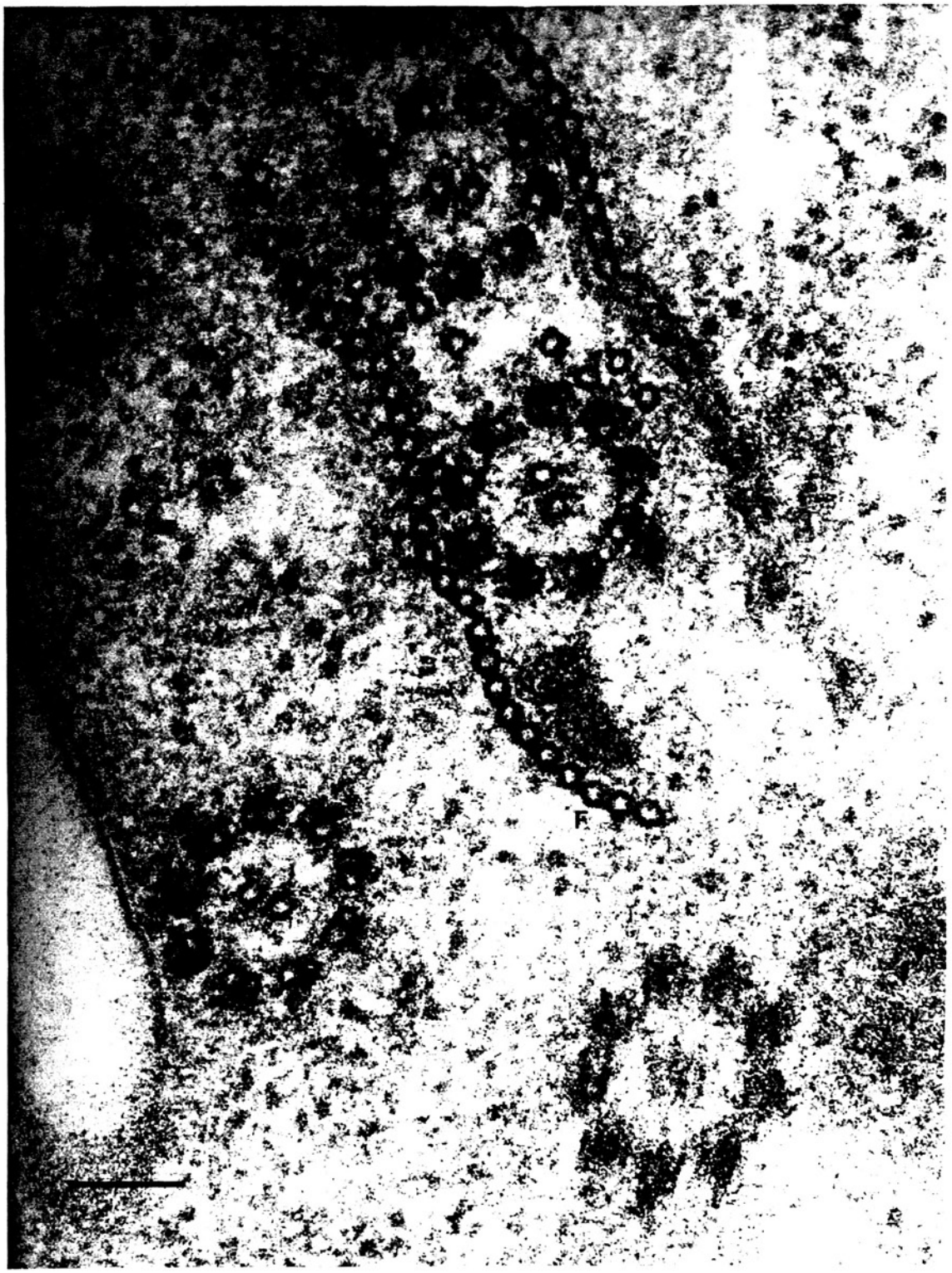


Photograph 6: Susceptible trophozoite exposed to 9 µM albendazole for 24 h. Bar = 1 µm.

Photograph 7: Enlargement of median body from a susceptible trophozoite after albendazole exposure. Bar = 0.1 $\mu$m.

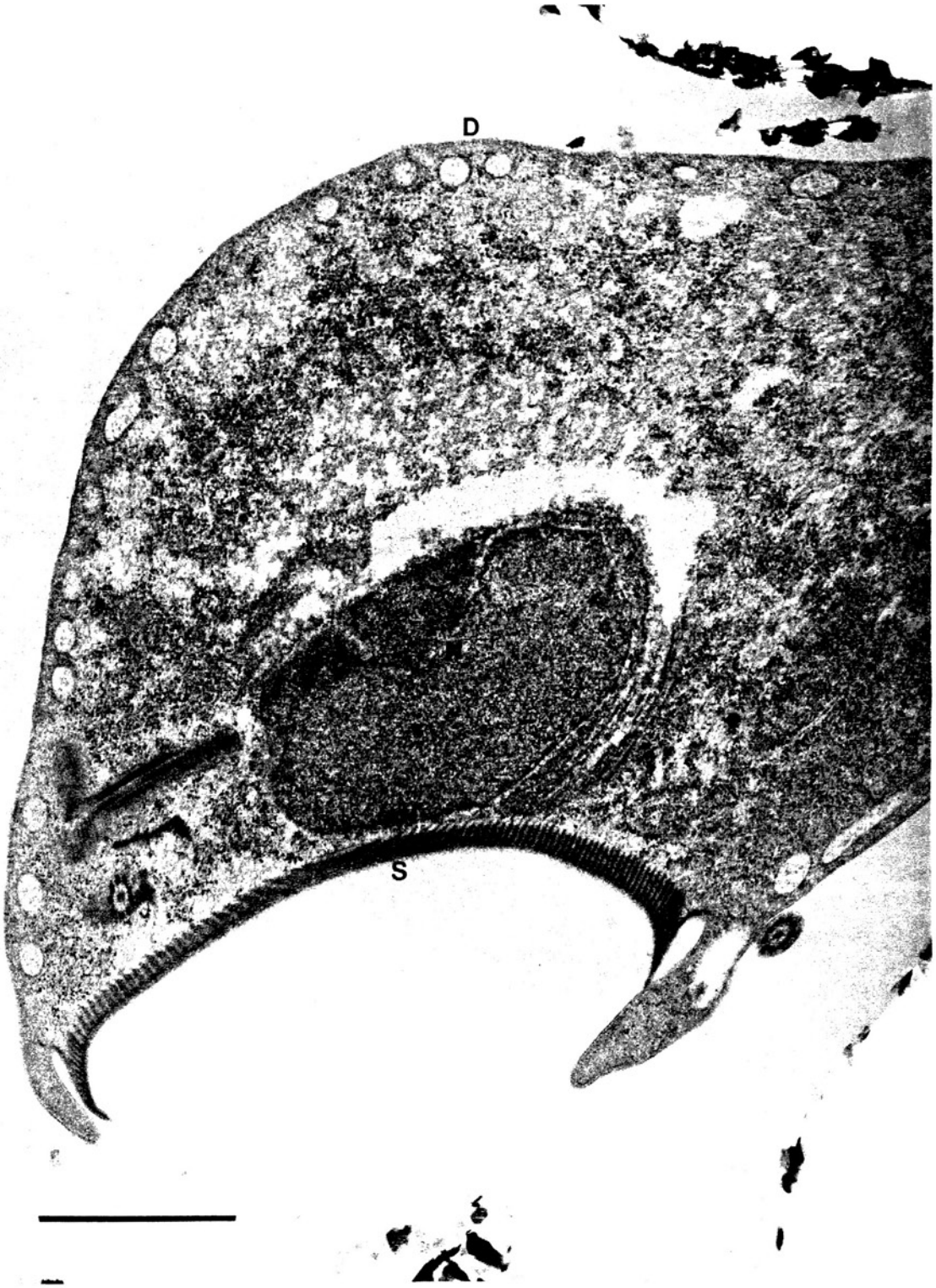


Photograph 8: Enlargement of funis from a susceptible trophozoite after albendazole exposure. Bar = 0.1 $\mu$m.



Photograph 9: Enlargement of striated disk from a susceptible trophozoite after albendazole exposure. Note damaged striated disk. Bar = 0.1 $\mu$m.

Photograph 10: Trophozoite from resistant culture, exposed to 9 µM for 24 h. Note intact striated disk. Bar = 1 $\mu$m.



Photograph 11: Trophozoite from resistant culture, exposed to 9 µM albendazole for 24 h. Note damaged striated disk. Bar = 1 µm.

Photograph 12: Trophozoite from resistant culture, exposed to 9 µM albendazole for 24 h. Bar = 1 µm.

Photograph 13: Enlargement of median body and funis from photograph 12. Bar = 0.5 $\mu$m.

Photograph 14: Enlargement showing funis and striated disk of photograph 12. Bar = 0.1 $\mu$m.

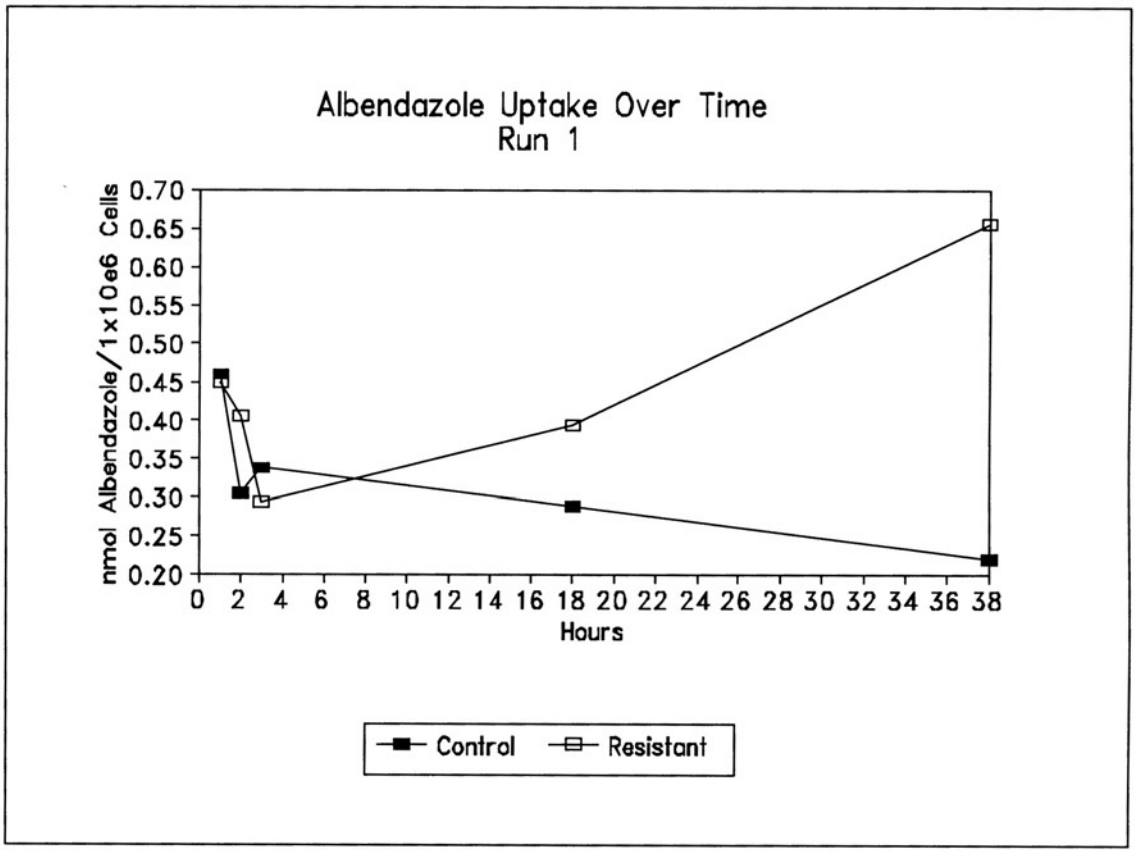
Photographs 10, 11 and 12 show trophozoites from a resistant population after exposure to 9 μM albendazole for 24 h. In photographs 10 and 12 there is no observable ultrastructural damage to the trophozoite, while photograph 11 shows some alteration of the striated disk. Photograph 12 shows the funis, median body, and striated disk. Photograph 13 is a greater magnification of the median body and funis of this trophozoite and photograph 14 shows the striated disk of this resistant trophozoite after exposure to albendazole.

## Radiolabeled Albendazole Uptake:

Data were first converted to nmol $^{14}$C-albendazole per 1X $10^6$ trophozoites, or nmol per ml where appropriate. Both resistant and susceptible *G. lamblia* took $^{14}$C-albendazole up from the medium. When compared to the supernate, there was between 1.5 and 25.7 (average 5.3) times as much albendazole in the pellet as would have been expected in an equal volume of the supernatant medium in the susceptible strain with 1.8 to 24.8 (average 6.4) times as much $^{14}$C-albendazole in the pellet compared to an equal volume of supernate.

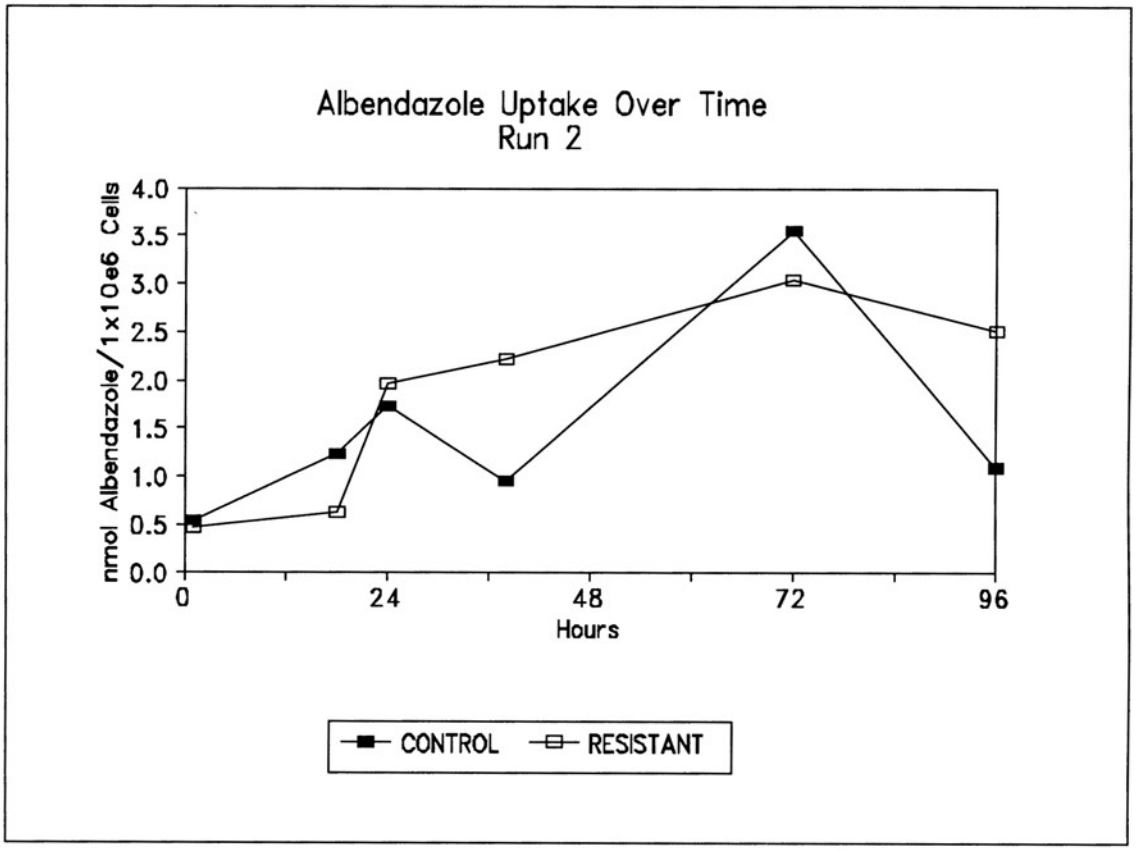
Data from $^{14}$C-albendazole uptake experiments are shown on figures 7, 8 and 9. Figure 7 represents the first trial run, figure 8 the second and figure 9 is a compilation of all data. These figures show that at 38 h there is a significant increase in the amount of $^{14}$C-albendazole taken up by resistant trophozoites when compared to controls.

Figure 7 Uptake of $^{14}C$-Labeled Albendazole by Resistant and Susceptible Strains (38 h).



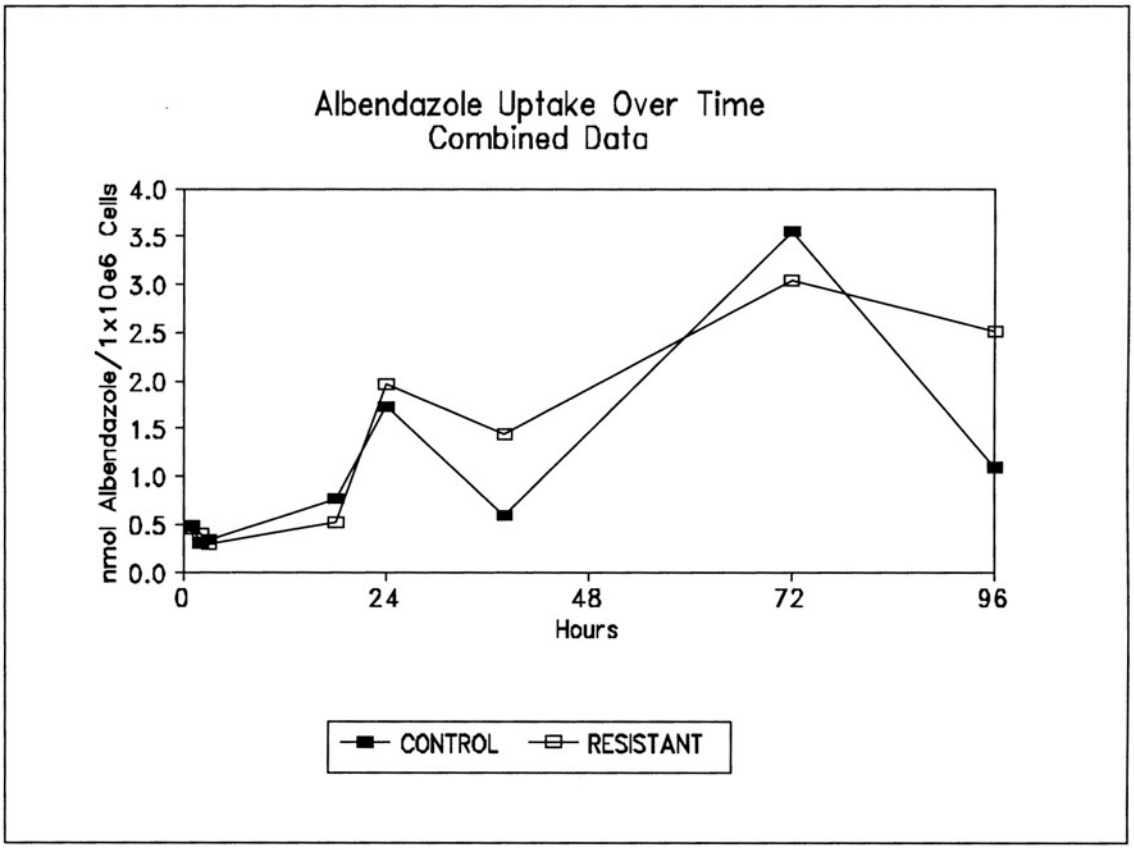

Uptake of radiolabeled albendazole given in nmol albendazole / $1x10^6$ trophozoites (Y axis) plotted over time (X axis). Uptake recorded over a 38 h time period.

Figure 8 Uptake of $^{14}C$-Labeled Albendazole by Resistant and Susceptible Strains (96 h).




Uptake of radiolabeled albendazole given in nmol albendazole / $1x10^6$ trophozoites (Y axis) plotted over time (X axis). Uptake recorded over a 96 h time period.

Figure 9 Uptake of $^{14}C$-Labeled Albendazole by Resistant and Susceptible Strains (Pooled Data).




Uptake of radiolabeled albendazole given in nmol albendazole / $1x10^6$ trophozoites (Y axis) plotted over time (X axis). Uptake data pooled for 38 h and 96 h runs.

These graphs also show the maximum accumulation of $^{14}C$-albendazole of about 3 nmol/$10^6$ trophozoites after 72 h exposure.

Data for both trial runs were analyzed by analysis of variance, a general linear model and regression analysis. Analysis of variance showed a significant difference between resistant and susceptible strains ($p<0.026$) when time and trial run were considered. Analysis of variance in this case may not be appropriate because not all measured time periods had the same number of observations. The general linear model and regression analysis showed no significant difference between resistant and susceptible strains ($p>0.305$, and $p>0.368$, respectively).

There was significant inter-run variability between trial runs of albendazole. Two factors were thought to influence the inter-run variability. The first was the length of time since previous exposure to albendazole. The first run was performed on trophozoites which had not been challenged by albendazole exposure for more than one week. The second run was accomplished one week after exposure. Also, the second run included a longer time frame. The reason for the difference in the time points picked for measurement of uptake is described in the methods section. When the data for the second run was graphed independently it was seen that the 72 and 96 h time periods represented a plateau of uptake in the resistant strain.

Given these two effects, the general linear model was adjusted to reflect the factors influencing the model. When the effect of trial run was absorbed into the variable for strain (resistant or susceptible) and eliminating the data representing the plateau (all data beyond 38 h) the general linear model was significant ($p<0.001$) with strain (resistant or susceptible) grouped by hour significant as well ($p<0.05$).

When the observation at 38 h was compared by T-testing for each run between resistant and susceptible strains there were significant differences in each run ($p>0.01$).

**Discussion:**

Induction of Resistance:

This study suggests that albendazole resistance could arise in nature. The results of this study show that *G. lamblia* has the ability to develop resistance to albendazole exposure at least 3.7 times higher than the previously reported minimum lethal concentration for *G. lamblia in vitro*. Efforts should be made to isolate and characterize *G. lamblia* from individuals who have *G. lamblia* that is refractory to treatment by albendazole to determine if there are naturally occurring populations of albendazole resistant parasites already.

Efforts to characterize the drug resistance induced in *G. lamblia* in the laboratory have led to two possible explanations for the mechanism of resistance: altered permeability of *G. lamblia* to albendazole (either through decreased uptake or through increased expulsion of the drug), or an altered target of action in the parasite similar to the mechanisms seen in albendazole resistance in helminths.

Stability of Resistance:

Stability of resistance over time without exposure to recurrent drug challenge is probably no greater for albendazole resistance than for metronidazole resistance. This is consistent with the concept that the genetic plasticity inherent in *G. lamblia* leads to the rapid development of resistance (Upcroft and Upcroft, 1993), and perhaps the rapid loss of resistance, in the absence of drug selection pressure.

Drug Inactivation:

The cross inoculation of susceptible cultures with medium containing albendazole from resistant cultures led to the death of the susceptible cultures. It is unlikely that albendazole is either inactivated, or sequestered in a biologically unavailable form by resistant trophozoites.

Encystation Ability:

Albendazole has been seen by electron microscope to precipitate around the structural elements of susceptible *G. lamblia* trophozoites, particularly associated with tubulin and structural components of the striated disk (Chavez, 1992). If the mechanism of resistance involves an alteration, or alterations to these proteins, then it may be expected that encystation efficacy may be impaired in drug resistant *G. lamblia*. Encystation trials showed that resistant trophozoites were in fact capable of encystation. Since encystation is not 100% efficient in susceptible populations and given the difficulty in quantifying of encystation, encystation efficiency was not calculated. Cysts were differentiated on the basis of morphology, and classified as Type I, Type II, and other. Type I cysts have a high index of refraction on examination under Nomarski DIC, cytosolic material which appears to fill the entire cyst, and recognizable internal structures. In type II cysts the cellular material appears to be pulled back from the cyst wall, and there are fewer or no recognizable internal structures. Type I cysts were observed in both susceptible and resistant populations of *G. lamblia*. This indicates that the development of drug resistance does not destroy the ability of *G. lamblia* to encyst. This result further implies that the structural proteins necessary for the process of encystation retain their function, and any

alteration of the structural proteins during the development of resistance does not affect encystation ability.

Cross Resistance to Metronidazole:

The lack of cross resistance to metronidazole conferred by albendazole resistance suggests different mechanisms of resistance for these two drugs. The resistance mechanism of *G. lamblia* to metronidazole involves either an alteration in transmembrane transport, or a reduced reliance on the PFOR pathway. It was thought to be unlikely that the PFOR pathway would play any role in albendazole resistance, but that there might be a possibility that a transport mechanism might be effective in both albendazole and metronidazole resistance. Since there is no apparent cross resistance for metronidazole in this particular albendazole resistant *G. lamblia* leads to the conclusion that the mechanisms of resistance for these two drugs are probably unrelated.

Continuous Exposure to Albendazole:

The killing of resistant trophozoites by prolonged exposure to albendazole could be attributable to more than one explanation. One possibility is that transport is altered in resistant trophozoites, either by reduced uptake of albendazole, or by increased expulsion. Another possible explanation for the killing of resistant trophozoites by prolonged exposure is that there is an alteration of the

tubulin structure, or the structure of other proteins. Albendazole may work by binding to and depolarizing tubulin; thus a decrease in the affinity of tubulin for albendazole (Horton *et al.*, 1990, Roos, 1990) or a transient decrease of the concentration of albendazole inside the trophozoite would both be possible mechanisms for resistance which can be overcome by prolonged exposure to albendazole.

If the mechanism of resistance is a passive reduced permeability to albendazole, then this mechanism is not 100% efficient, at least at this level of resistance. If the mechanism of resistance involves active transport, then the mechanism may overwhelmed by the constant drug pressure.

An alternative explanation is that the site of activity of the drug is altered. If this is true, albendazole either slowly overcomes this mechanism, or has a secondary mechanism of action. In the first scenario a lower binding affinity of giardins and tubulin for albendazole might protect the trophozoite in a short term exposure. In a long term exposure the drug would still be able to bind in sufficient quantity to the target structural components to cause cell death and inhibit replication.

Another scenario would imply that albendazole might bind to tubulin or giardins and kill the cell as a primary mechanism of action. In resistant trophozoites, the target of action would have been altered to confer resistance, yet albendazole would still have the ability to kill *G. lamblia* through some other mechanism. In this instance resistant

parasites might have an altered tubulin which prevents binding of albendazole, yet the albendazole retains its activity through its interruption of giardins. To test these hypotheses, the ultrastructural properties of resistant and susceptible trophozoites exposed to 9 μM, 24 h albendazole, and the ability of resistant and susceptible trophozoites to take $^{14}C$-labeled albendazole up from medium were studied.

Ultrastructural Examination:

A number of trophozoites which are resistant to a 9 μM, 24 h exposure to albendazole show no obvious disruption of the striated disk, even though some appear to have lost some of their typical morphology and are bulbous. The presence of trophozoites with either disrupted or intact striated disks may represent a heterogeneous population of susceptible and resistant trophozoites in the resistant population. Major ultrastructural features comprised of tubulin: the flagella, axonemes, funis, and median bodies appear to be intact in at least some of the susceptible trophozoites which have been exposed to 9 μM albendazole for 24 h. In susceptible trophozoites, the major ultrastructural difference from unexposed and resistant trophozoites appears to be disruption of the striated disk and an altered relationship between the tubules of the disk and the ribbons of proteins which are attached to them.

## Radiolabeled Albendazole Uptake:

Radiolabeled albendazole is taken up from the medium by both resistant and susceptible strains of *G. lamblia*. After 38 h the rate of uptake of $^{14}$C-albendazole in resistant trophozoites is even greater than for susceptible trophozoites. There was no significant difference between the maximum amount of albendazole taken up from the medium between resistant and susceptible strains after 72 h exposure.

The increase in rate of accumulation of $^{14}$C-albendazole, coupled with the fact that many resistant trophozoites maintain an intact striated disk, is indicative of a process by which the resistant trophozoites increase the stability of their structural proteins. If the drug were detoxified, then there should be some reduction in the ability of the medium to kill susceptible trophozoites after exposure, since at 38 h the resistant trophozoites had sequestered 7.3% of the total amount of drug present in the medium (6.9 nmol in pellet of a total 94.5 nmol inoculated in the 10.5 ml culture). If the drug were not imported, or if it were exported from the trophozoite, a lower level of $^{14}$C-albendazole would have been expected in the resistant cultures, especially in the first 24 h.

**Conclusions:**

This is the first report of resistance to albendazole in *G. lamblia* induced in the laboratory. This report also confirms the observations of Kortbeek *et al.* (1994) who have isolated a strain of albendazole resistant *G. lamblia* from patients.

Drug resistance was first developed in this study using cyclical exposure to low doses of albendazole. It was determined that prolonged exposure to albendazole would kill trophozoites which were resistant to a 24 h exposure to the same dose. This model may be realistic since albendazole may be given in single doses at a sub-therapeutic level for giardiosis where prolonged doses may be curative (Hall and Nahar, 1993). The use of albendazole at sub-curative dosage for giardiosis for treatment of helminthic infections may make the development of resistance in field populations a likely occurrence (Coulaud and Rossignol 1984, Kollaritsch *et al.*, 1993, and Hall and Nahar, 1993). Since encystation ability is retained in resistant *G. lamblia* there is the possibility that albendazole resistant *G. lamblia* will be transmissible.

In a review of benzimidazoles, Horton *et al.* (1990) suggested two possible mechanisms for benzimidazole resistance. One possible mechanism for benzimidazole resistance is an alteration of the β-tubulin subunit of tubulin, resulting in the lack of ability of albendazole to

bind to this tubulin subunit. The second possible mechanism of resistance is that the benzimidazole is actively effluxed from the cell. Evidence that tubulin alteration is responsible for benzimidazole resistance in a variety of nematodes has been given by a number of authors (Enos and Coles, 1990, Lubega and Prichard, 1990, and Roos, 1990).

Morgan *et al.* (1993) noted that the morphology of trophozoites exposed to albendazole was greatly altered, but the flagella, which are comprised mainly of microtubules, remained in motion. Chavez *et al.* (1992) demonstrated dark precipitate around the microtubules of albendazole exposed *G. lamblia* when compared to unexposed controls. They also found morphological changes and disruptions of the striated disk. In this study flagellar activity was observed in albendazole exposed control specimens confirming the work of Morgan *et al.* (1993), and while the structural damage to the striated disk was similar to that reported by Chavez *et al.* (1992) there seemed to be no electron dense deposition around the microtubules. The observation by Enos and Coles (1990) that *Caenorhabditis elegans* which were susceptible to albendazole demonstrated an absence of microtubules in intestinal tract cells also contrasts with that of both Chavez *et al.* (1992) and with this study in which microtubules are still evident in susceptible *G. lamblia* after exposure to albendazole.

Oxberry *et al.* (1994) also arrived at the conclusion that an effect of albendazole on the striated disk was

responsible for killing of *G. lamblia*. Oxberry *et al.* (1994) arrived at this conclusion by comparison of the results of exposure to albendazole on *G. lamblia*, with results of exposure on the closely related lumen dwelling protozoan parasites *Trichomonas vaginalis* and *Spironucleus muris*. Both of these parasites, although affected by albendazole to some extent, were not as severely affected as was *G. lamblia*. In *G. lamblia*, the effects of albendazole exposure in this study appeared to be mainly in the destruction of the striated disk. These data suggest that in *G. lamblia*, albendazole may have its main action against the striated disk structural proteins and is less effective against the tubulin of *G. lamblia*.

In the study undertaken here, the results of previous authors (Chavez *et al.*, 1992, Jarroll, 1994, and Oxberry *et al.*, 1994) are confirmed, namely that the apparent target of albendazole may in fact be the striated disk. In resistant trophozoites, the striated disk appears to remain intact.

The resistance developed in this study was of a transient nature. If exposed to prolonged doses of albendazole, trophozoites were killed even though they had been resistant to a 24 h exposure of the same dosage. This observation led to a preliminary hypothesis that resistance was due to the failure of transport of albendazole into the trophozoite, or an increased expulsion from the trophozoite. It was felt that a change in the structural proteins which

conferred resistance for a 24 h exposure, should be sufficient to confer resistance for longer periods as well. Hence $^{14}C$-labeled albendazole uptake studies were undertaken. In this model of resistance, the rate of accumulation of albendazole should have been lower in resistant trophozoites than susceptible. In malaria, resistance to antimalarial chemotherapeutic agents by active transport of these agents out of the parasite has been suggested (Macomber *et al.*, 1966, and Krogstad *et al.*, 1987). In *G. lamblia* a transport mechanism has been suggested for metronidazole resistance (Boreham *et al.*, 1988, and Upcroft *et al.*, 1994).

The $^{14}C$-albendazole uptake studies reported herein indicate that, not only do albendazole resistant *G. lamblia* trophozoites fail to exclude albendazole, but they actually incorporate more drug during the first 38 hours of exposure. The change in kinetics of drug uptake is indicative of a change in the protein to which the drug binds. The striated disk is seen to be disrupted in susceptible trophozoites, but remains intact in resistant trophozoites. It is probable therefore, that these proteins which have been altered to have increased stability in the presence of albendazole.

The implications of this study are that the use of albendazole for both giardiosis and helminthic infections is that drug dosage should be carefully considered so as to avoid the development of albendazole resistance in *G.*

*lamblia*. The possibility of developing albendazole resistant *G. lamblia* is serious since albendazole is regularly given in doses which are sub-therapeutic for *G. lamblia* during treatment for helminthic infections of humans.

In cases where helminthic infection is to be treated with albendazole, either a dose of albendazole to which is curative for *G. lamblia* might be used, or an exhaustive examination to rule out concurrent *G. lamblia* infection should be considered. Since *G. lamblia* infections may be inapparent, especially in asymptomatic cases, it may be advisable to adjust the dosage of albendazole such that it is a sufficient curative dose for *G. lamblia* . This of *course must be moderated by the constraints of good medical* practice to limit the amount of unnecessary treatment.

The presence of animal reservoirs of *G. lamblia* poses further problems. In those animals known to harbor *G. lamblia* which may be potential sources of infection for humans, the implications of the possibility of development of albendazole resistant *G. lamblia* should be considered when albendazole is to be used for treatment of helminthic infections. This may require that studies be done on domestic livestock which can harbor *G. lamblia* capable of infecting humans, to determine the effective dose of albendazole for curing *G. lamblia* infection. This dosage could then be used as a minimum recommendation for treatment of any helminthic infection, to avoid development of

albendazole resistant *G. lamblia* in the livestock population which might then be transmitted to humans.

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**Appendix 1**

**KEISTER'S MODIFIED TYI-S-33 MEDIUM:**

(Adapted from Cote *et al.*, 1984)

| Component | amt./l |
|---|---|
| Biosate (BBL) | 30.0 g |
| Dextrose | 10.0 g |
| Bovine Bile | 0.75 g |
| NaCl | 2.0 g |
| L-Cysteine HCl | 2.0 g |
| Ascorbic Acid | 0.2 g |
| $K_2HPO_4$ | 1.0 g |
| $KH_2PO_4$ | 0.6 g |
| Ferric Ammonium Citrate | 22.8 mg |
| $dH_2O$ (glass distilled) | 750. ml |

Adjust pH to 7.0-7.2 with 1N NaOH. Add:

| | |
|---|---|
| Bovine Serum | 100. ml |
| Diamond's Tween 80 Vitamin Mixture | 30. ml |

Aseptically filter sterilize, first through 0.45 µM, then through 0.22µM membrane filter. Store at 4°C. Aseptically dispense 13 ml or 22 ml complete medium in 16 x 125 mm or 20 x 125 mm screw capped borosilicate glass culture tubes, or store in 500 ml bottle and aliquot at time of use.

**Appendix 2** **GEL FORMULATIONS:**

## Polyacrylamide Gel

( from Laemelli, 1970)

| COMPONENT | AMOUNT | |
|---|---|---|
| **Stock A** | | |
| Acrylamide | 56.0 | g |
| Bis-acrylamide | 1.47 | g |
| Distilled-$H_2O$ | 200. | ml |
| **Solution A** | | |
| Tris base | 73.2 | g |
| 1N HCl | 96.0 | ml |
| TEMED | 0.46 | ml |
| Distilled-$H_2O$ | 200. | ml |
| **Solution D** | | |
| 1M Tris base pH 7.0 | 49.5 | ml |
| TEMED | 0.125 | ml |
| **Ammonium Persulfate** | | |
| Ammonium persulfate | 0.035 | g |
| Distilled-$H_2O$ | 25. | ml |

Make up fresh, the day of use.

## Running Gel

| | 15.% | 12.% | 7.5% | 5.% |
|---|---|---|---|---|
| (For small gels) | | | | |
| Stock A | 5.45 ml | 4.26 ml | 2.66 ml | 1.82 ml |
| Distilled $H_2O$ | 0.55 ml | 1.74 ml | 3.34 ml | 4.18 ml |
| Solution A | 1.32 ml | 1.32 ml | 1.32 ml | 1.32 ml |
| Ammonium persulfate | 2.63 ml | 2.63 ml | 2.63 ml | 2.63 ml |
| (For large gels) | | | | |
| Stock A | 20.7 ml | 16.2 ml | 10.12 ml | 6.9 ml |
| Distilled $H_2O$ | 2.1 ml | 6.6 ml | 12.67 ml | 15.9 ml |
| Solution A | 5.0 ml | 5.0 ml | 5.0 ml | 5.0 ml |
| Ammonium persulfate | 10.0 ml | 10.0 ml | 10.0 ml | 10.0 ml |

## Stacking Gel

| | |
|---|---|
| Stock A | 3.0 ml |
| Distilled $H_2O$ | 5.0 ml |
| *Solution D* | 4.0 ml |
| Ammonium persulfate | 16.0 ml |

## Starch Gel

(From Dr. Sylvie Gargarine)

| | | |
|---|---|---|
| Gel Buffer | 400.0 | ml |
| Electrostarch | 25.0 | g |
| Sigma Starch | 25.0 | g |
| Sucrose (Optional) | 20.0 | g |

Heat with constant swirling in necked erlenmeyer flask. When bubbles rise from bottom (starch completely dissolved) remove from heat, degass (capped, vacuum) 4-5 min. Pour. Cover with plastic wrap leave at room temp. overnight or refrigerate a few hours.

## Agarose Gel

Make gels with appropriate percentage of agarose in appropriate buffer system. Heat to melt agarose, with swirling. Degass briefly. Pour.

**Appendix 3**

## PHOSPHATE BUFFERED SALINE (PBS):

Adapted from Melvin and Brooke (1982)

Phosphate Buffered Saline pH 7.4 (1X)

Sodium Chloride.....8.0 g

$KH_2PO_4$.....0.2 g

$Na_2HPO_4$.....2.9 g

KCl.....0.2 g

distilled $H_2O$ q.s. to .....1000 ml

Dissolve solid ingredients into about 800 ml $dH_2O$. Adjust pH to 7.4, and adjust volume to 1000 ml. For use, dilute 1:10 with distilled water and filter sterilize if required.

**Appendix 4**

**COOMASSIE BRILLIANT BLUE G-250 PROTEIN DETERMINATION:**

Use 0.8 ml protein solution, 0.2 ml biorad dye reagent concentrate. Mix in optically clear test tube, and allow to react for 5-15 minutes before reading in a spectrophotometer at 595 nm.

For each series of protein determinations, a standard curve was constructed using as a standard, a known concentration of protein. The protein used was bovine serum albumen, rehydrated from lyophilized crystalline powder. Buffer solution without protein, as well as 5 concentrations of protein from 1 µg per ml to 120 µg per ml were used as standards. The absorbency data was then plotted a graph created in Quatro Pro $^{tm}$. Then the absorbencies of the unknowns to be read were plotted on the same graph and the concentrations of protein in these samples read off the graph.

**Appendix 5**

**EXCYSTATION PROTOCOLS:**

(Boucher and Gillin, 1990)

I. Prepare Stock Solutions

A. Prepare HBSS with L-cysteine and glutathione (10X).

Hanks' Balanced Salt Solution (HBSS) 10X Stock (from EPA SOP).

| | 1l | 100ml |
|---|---|---|
| $dH_2O$ | 750 ml | 75 ml |
| NaCl | 80 g | 8 g |
| KCl | 4 g | 0.4 g |
| $MgSO_4$ $7H_2O$ | 1 g | 0.1 g |
| $Na_2HPO_4$ | 600 mg | 60 mg |
| $KH_2PO_4$ | 600 mg | 60 mg |
| $MgCl_2$ $6H_2O$ | 1 g | 0.1 g |
| $CaCl_2$ | 1.4 g | 140 mg |
| Glucose | 10 g | 1 g |

a) Place 750 ml (75 ml) $dH_2O$ into a clear glass vessel.

b) Maintain constant stirring.

c) Add ingredients to the water in order listed.

**ALLOW EACH INGREDIENT TO GO INTO SOLUTION BEFORE ADDING THE NEXT ONE.**

d) Adjust volume of solution to 1 l (100 ml) with $dH_2O$.

e) Filter sterilize medium through a 0.22 µm membrane.

f) Store at 4°C.

B. Prepare Tyrode salt solution (20X). (Schaefer *et al.*, 1980.)

Tyrode's Solution 20X

| | |
|---|---|
| NaCl | 160 g |
| KCl | 4 g |
| $CaCl_2$ | 4 g |
| $MgCl_2$ $6H_2O$ | 2 g |
| $NaH_2PO_4$ $H_2O$ | 1 g |
| $NaHCO_3$ | deleted |
| Glucose | 20 g |
| $dH_2O$ | 1 l |

Tyrode's solution 1X dilute 5 ml of the 20X stock solution to 100 ml with distilled water (q.s. in volumetric flask).

C. Keister's modified TYI-S-33 with antibiotics. (See SOP). Add 125 µg/ml amikacin and 500 µg/ml piperacillin (Boucher and Gillin 1990).

II. Protocol A (low pH induction medium with L-cysteine and glutathione, and proteinase medium).

A. Prepare working solutions.

1. Prepare low pH induction medium.

a. Prepare 0.1M $NaHCO_3$.

**0.1 M $NaHCO_3$**

$dH_2O$.....10 ml

$NaHCO_3$..... 0.84 g

1) Use only cold water.

2) Mix constantly.

3) Mix until sodium bicarbonate is completely dissolved.

b. Prepare HBSS with glutathione and L-cysteine.

1) Dilute 1 ml HBSS to 10 ml with $dH_2O$ for use.

2) Add:

0.1 g glutathione (reduced form) crystalline.

0.1 g L-cysteine-HCl monohydrate to 10 ml 1X HBSS.

c. Use 6.8 ml of 1 X HBSS with L-cysteine and glutathione.

d. Add 6.8 ml (0.1 M) $NaHCO_3$.

e. Add 11.3 ml $dH_2O$

f. Adjust pH of the solution to 4.0 with 0.01 N HCl.

g. Store chilled but use the same day. Prewarm before using.

2. Prepare proteinase medium.

a. Prepare fresh 7.5% sodium bicarbonate solution.

**7.5% $NaHCO_3$**

| | 100 ml | 10 ml |
|---|---|---|
| $dH_2O$ | 100 ml | 10 ml |
| $NaHCO_3$ | 7.5 g | 0.75 g |

1) Use only cold water.

2) Mix constantly.

3) Mix until sodium bicarbonate is completely dissolved.

4) Store chilled for use the same day.

b. Prepare 1X Tyrode's solution (5 ml 20X diluted to 100 ml with $dH_2O$).

c. Weigh out 0.5 g 1:100 trypsin and 0.15 g $NaCHO_3$ into a 150 ml or larger beaker.

d. Slowly add Tyrode's solution to dry reagents while mixing.

e. Mix continuously on magnetic stir-plate for 30 min.

f. Spin at 21,000 x g for 10 min.

g. Filter the supernate through a 0.45 µm membrane using positive pressure.

h. Adjust pH of Tyrode-Trypsin to pH 8.0 with 7.5% $NaHCO_3$.

C. Expose cysts to low pH induction medium.

1. Mix 50 µl cyst suspension and 0.55 ml low pH induction medium in an Eppendorf

tube. Cyst suspension should contain between $5X10^3$ to $5X10^4$ cysts. **(About 1 ml/cysts to about 9 ml induction medium.)**

2. Incubate induction medium cyst suspension in a 37.5 °C water bath for 30 min.

3. Prewarm proteinase mixture.

4. Sediment cysts at 135 x g and aspirate and discard supernate. **(600 x g 10 min. )**

D. Expose cysts to proteinase medium.

1. Add 1 ml prewarmed proteinase mixture containing 50 U/mg protein in Tyrode salt solution with pH raised to 8.0 with freshly prepared 7.5% sodium bicarbonate. [Trypsin is used as per Schaefer *et al*. (1980).]

2. Incubate cyst pellet with proteinase mixture for 30 min in a 37.5 °C water bath.

3. Prewarm TYI-S-33 with antibiotics.

4. Sediment for 3 min at 135 x g and aspirate and discard supernate. (600 x g 10 min.)

5. Add 100 µl prewarmed BI-S-33 (with antibiotics). (Inoculate pellet into BI-S-33 containing antibiotics.)

III. Protocol B (low pH induction medium with L-cysteine only, and proteinase medium).

A. Prepare working solutions.

1. Prepare low pH induction medium.
    a. Prepare 0.1M $NaHCO_3$ as above.
    b. Prepare HBSS with and L-cysteine.
        1) Dilute 1 ml HBSS to 10 ml with $dH_2O$ for use.
        2) Add:
        0.1 g L-cysteine-HCl monohydrate to 10 ml 1X HBSS.
    c. Use 6.8 ml of 1 X HBSS with L-cysteine.
    d. Add 6.8 ml (0.1 M) $NaHCO_3$.
    e. Add 11.3 ml $dH_2O$
    f. Adjust pH of the solution to 4.0 with 0.01 N HCl.
    g. Store chilled but use the same day. Prewarm before using.
2. Prepare proteinase medium.
    a. Prepare fresh 7.5% sodium bicarbonate solution as above.
    b. Prepare 1X Tyrode's solution (5 ml 20X diluted to 100 ml with $dH_2O$).
    c. Weigh out 0.5 g 1:100 trypsin and 0.15 g $NaCHO_3$ into a 150 ml or larger beaker.
    d. Slowly add Tyrode's solution to dry reagents while mixing.

e. Mix continuously on magnetic stir plate for 30 min.

f. Spin at 21,000 x g for 10 min.

g. Filter the supernate through a 0.45 µm membrane using positive pressure.

h. Adjust pH of Tyrode-Trypsin to pH 8.0 with 7.5% $NaHCO_3$.

C. Expose cysts to low pH induction medium.

1. Mix 50 µl cyst suspension and 0.55 ml low pH induction medium in an Eppendorf tube. Cyst suspension should contain between $5X10^3$ to $5X10^4$ cysts. (About 1 ml/cysts to about 9 ml induction medium.)

2. Incubate induction medium cyst suspension in a 37.5 °C water bath for 30 min.

3. Prewarm proteinase mixture.

4. Sediment cysts at 135 x g and aspirate and discard supernate. (600 x g 10 min. )

D. Expose cysts to proteinase medium.

1. Add 1 ml prewarmed proteinase mixture containing 50 U/mg protein in Tyrode salt solution with pH raised to 8.0 with freshly prepared 7.5% sodium bicarbonate. (Trypsin is used as per Schaefer *et al.* 1980.)

2. Incubate cyst pellet with proteinase mixture for 30 min in a 37.5 °C water bath.

3. Prewarm TYI-S-33 with antibiotics.

4. Sediment for 3 min at 135 x g and aspirate and discard supernate. (600 x g 10 min.)

5. Add 100 µl prewarmed TYI-S-33 (with antibiotics). (Inoculate pellet into TYI-S-33 containing antibiotics.)

IV. Protocol C (low pH induction medium with L-cysteine only and no proteinase medium).

A. Prepare working low pH induction medium.

1. Prepare 0.1M $NaHCO_3$ as above.

2. Prepare HBSS with and L-cysteine.

a. Dilute 1 ml HBSS to 10 ml with $dH_2O$ for use.

b. Add:

0.1 g L-cysteine-HCl monohydrate

to 10 ml 1X HBSS.

3. Use 6.8 ml of 1 X HBSS with L-cysteine.

4. Add 6.8 ml (0.1 M) $NaHCO_3$.

5. Add 11.3 ml $dH_2O$

6. Adjust pH of the solution to 4.0 with 0.01 N HCl.

7. Store chilled but use the same day. Prewarm before using.

B. Expose cysts to low pH induction medium.

1. Mix 50 µl cyst suspension and 0.55 ml low pH induction medium in an Eppendorf

tube. Cyst suspension should contain between $5X10^3$ to $5X10^4$ cysts. (About 1 ml/cysts to about 9 ml induction medium.)

2. Incubate induction medium cyst suspension in a 37.5 °C water bath for 1 h.

3. Prewarm TYI-S-33 with antibiotics.

4. Sediment for 3 min at 135 x g and aspirate and discard supernate. (600 x g 10 min.)

5. Add 100 µl prewarmed TYI-S-33 (with antibiotics). (Inoculate pellet into TYI-S-33 containing antibiotics.)

V. Protocol D (no low pH induction medium, proteinase medium only).

A. Prepare working proteinase medium.

1. Prepare fresh 7.5% sodium bicarbonate solution as above.

2. Prepare 1X Tyrode's solution (5 ml 20X diluted to 100 ml with $dH_2O$).

3. Weigh out 0.5 g 1:100 trypsin and 0.15 g $NaCHO_3$ into a 150 ml or larger beaker.

4. Slowly add Tyrode's solution to dry reagents while mixing.

5. Mix continuously on magnetic stir plate for 30 min.

6. Spin at 21,000 x g for 10 min.

7. Filter the supernate through a 0.45 µm membrane using positive pressure.

8. Adjust pH of Tyrode-Trypsin to pH 8.0 with 7.5% $NaHCO_3$.

9. Prewarm proteinase mixture.

B. Expose cysts to proteinase medium.

1. Add 1 ml prewarmed proteinase mixture containing 50 U/mg protein in Tyrode salt solution with pH raised to 8.0 with freshly prepared 7.5% sodium bicarbonate. (Trypsin is used as per Schaefer *et al*. 1980.) (1 ml cyst suspension and 9 ml proteinase medium.)

2. Incubate cyst pellet with proteinase mixture for 1 h in a 37.5 °C water bath.

3. Prewarm TYI-S-33 with antibiotics.

4. Sediment for 3 min at 135 x g and aspirate and discard supernate. (600 x g 10 min.)

5. Add 100 µl prewarmed TYI-S-33 (with antibiotics). (Inoculate pellet into TYI-S-33 containing antibiotics.)

A. Prepare working solutions.

1. Prepare 0.1M $NaHCO_3$ as above.

b. Prepare HBSS with glutathione and L-cysteine.

1) Dilute 1 ml HBSS to 10 ml with $dH_2O$ for use.

2) Add:

0.1 g glutathione (reduced form) crystalline.

0.1 g L-cysteine-HCl monohydrate to 10 ml 1X HBSS.

2. Prepare low pH induction medium with L-cysteine and glutathione (protocol A).

c. Use 6.8 ml of 1 X HBSS with L-cysteine and glutathione.

d. Add 6.8 ml (0.1 M) $NaHCO_3$.

e. Add 11.3 ml $dH_2O$

f. Adjust pH of the solution to 4.0 with 0.01 N HCl.

g. Store chilled but use the same day. Prewarm before using.

3. Prepare low pH induction medium with L-cysteine only (protocol B and C).

b. Prepare HBSS with and L-cysteine.

1) Dilute 1 ml HBSS to 10 ml with $dH_2O$ for use.

2) Add:

0.1 g L-cysteine-HCl monohydrate to 10 ml 1X HBSS.

c. Use 6.8 ml of 1 X HBSS with L-cysteine.

d. Add 6.8 ml (0.1 M) $NaHCO_3$.

e. Add 11.3 ml $dH_2O$

f. Adjust pH of the solution to 4.0 with 0.01 N HCl.

g. Store chilled but use the same day. Prewarm before using.

4. Prepare proteinase medium.

a. Prepare fresh 7.5% sodium bicarbonate solution as above.

b. Prepare 1X Tyrode's solution (5 ml 20X diluted to 100 ml with $dH_2O$).

c. Weigh out 0.5 g 1:100 trypsin and 0.15 g $NaCHO_3$ into a 150 ml or larger beaker.

d. Slowly add Tyrode's solution to dry reagents while mixing.

e. Mix continuously on magnetic stir plate for 30 min.

f. Spin at 21,000 x g for 10 min.

g. Filter the supernate through a 0.45 µm membrane using positive pressure.

h. Adjust pH of Tyrode-Trypsin to pH 8.0 with 7.5% $NaHCO_3$.

C. Expose cysts to low pH induction medium (protocols A, B, and C).

1. Mix 50 µl cyst suspension and 0.55 ml low pH induction medium in an Eppendorf

tube. Cyst suspension should contain between $5X10^3$ to $5X10^4$ cysts. (About 1 ml/cysts to about 9 ml induction medium.)

2. Incubate induction medium cyst suspension in a 37.5 °C water bath for 30 min (protocol A and B) or 1 hr (protocol D).

3. Prewarm proteinase mixture (protocols A and B).

4. Sediment cysts at 135 x g and aspirate and discard supernate. (600 x g, 4°C, 10 min. )

D. Expose cysts to proteinase medium (protocols A, B, and D).

1. Add 1 ml prewarmed proteinase mixture containing 50 U/mg protein in Tyrode salt solution with pH raised to 8.0 with freshly prepared 7.5% sodium bicarbonate. (Trypsin is used as per Schaefer *et al*. 1980.)

2. Incubate cyst pellet with proteinase mixture for 30 min in a 37.5 °C water bath (protocols A and B) or 1 h in a 37.5 °C (protocol D).

3. Prewarm TYI-S-33 with antibiotics.

4. Sediment for 3 min at 135 x g and aspirate and discard supernate. (600 x g 10 min.)

5. Add 100 µl prewarmed TYI-S-33 (with antibiotics). (Inoculate pellet into BI-S-33 containing antibiotics.)

**Alternate Excystation Procedure:**

(Isaac-Renton, *et al.*, 1986.)

I. Prepare Stock Solutions

II. Protocol (low pH induction medium with L-cysteine and proteinase and bile medium).

A. Prepare working solutions.

1. Dilute 1 ml Hanks (10X stock solution) to 10 ml with $dH_2O$.
2. Prepare 0.1M sodium bicarbonate (0.084 g $NaHCO_3$ in 10 ml $H_2O$).
3. Prepare aqueous HCl. Dilute HCl to a pH of 2.0. (Approx. 0.1 ml in 100 ml $H_2O$.)
4. Prepare trypsin-Tyrode.
   a. Dilute 1.25 ml Tyrode's to 25 ml with $dH_2O$.
   b. Add 50 mg trypsin.
   c. Add
   c. Warm to 37°C.
5. Pre-warm all solutions.

B. For 0.5 ml cysts.

1. Add 5 ml 1 mM HCl pH 2.0

2.5 ml Hanks

2.5 ml 0.1M $NaHCO_3$

2. Incubate at 37°C for 30 min.
3. Spin at 600 g for 10 min. Decant supernate.
4. Suspend in 10 ml trypsin-Tyrode solution (50 mg trypsin in 25 ml 1X Tyrode solution).
5. Spin at 600 g for 10 min. Decant supernate.
6. Re-suspend in 4 ml trypsin-Tyrode solution, add 2 ml of a 1:2 dilution of bovine bile (from fresh bile) and vortex.
7. Incubate 30 min at 37°C.
8. Spin at 600 g for 10 min. Decant supernate.
9. Excystation monitoring.
   a. *In vitro* excystation.
      i. Add 100 IU penicillin and 100 µg streptomycin per ml (1 ml Pen-Strep.) to 10 ml TYI-S-33 in a 13x120 mm screw capped borosilicate culture tube.
      ii. Suspend cyst pellet in TYI-S-33 (with Pen-Strep.) (about 1 ml).

iii. Put a drop in a depression slide, cover with a coverslip and seal with paraffin. Incubate for 15 min and examine slide for excystation.

iv. Incubate tube with the rest of the medium for 7-10 days monitoring periodically for excystation.

**Appendix 6**

**PROCEDURE FOR FLUORESCENT ANTIBODY STAINING:**

1. Prepare teflon coated 12 well slides by autoclaving to assure that the teflon coating sticks to the slide.
2. Prepare wells by adding *Giardia* culture. Cultures should be concentrated and concentration adjusted such that a good monolayer is formed by a drop (about 5,000 trophozoites/ 10 µl) of suspension. Add 10 µl culture and incubate at 20°C until dry about 2-3 hr or overnight.
3. Store slides in freezer (0°C). until use. Remove from freezer about 1 hr before use and warm to 20°C.
4. Prepare sample dilutions with PBS. Be sure to include positive and negative controls (negative control diluted 1:200 with PBS).
5. Draw sample distribution in lab book. Label slides.
6. Flood wells with 20 µl per well diluted sample.
7. Place slides in humid box (slide box with moist towel). Incubate 37°C, 30 min.
8. Dilute GAM Ab 1:40 in PBS, enough to use 20 µl per well for all wells to be stained, plus extra for wetting. Spin in microcentrifuge on high for 5 min.
9. Dip briefly in wash solution (PBS - 0.3% Tween 20).
10. Rinse in wash solution, 5 min.
11. Flood each well used with diluted conjugated second antibody prepared above, 20 µl per well.

12. Incubate in humid box, 30 min, 37°C.

13. Dip briefly in wash solution.

14. Rinse in wash solution 5 min.

15. Dry, pat dry on a paper towel.

16. Add 40% glycerol, 2 µl per well, every well (even those which are unstained).

17. Cover with coverslip.

18. Read under fluorescent microscope.

19. Store in refrigerator in the dark, should be read the same day if possible or within 24 h if necessary. GAM will be quenched usually within 1 week. Record results in lab book.